IDENTIFICATION OF EXPRESSION PROFILES OF SORGHUM GENES IN RESPONSE TO GREENBUG PHLOEM-FEEDING & CHARACTERIZATION OF THE TWO SORGHUM DEFENSE-RELATED GENES, *Xa1* AND *OXYSTEROL-BINDING PROTEIN*

BY

SUNG-JIN PARK

Bachelor of Arts
Korea University
Seoul, Korea
1997

Master of Science
Korea University
Seoul, Korea
1999

Submitted to the Faculty of the
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Oklahoma State University
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Dissertation Approved:

Dr. Yinghua Huang

Dissertation Advisor

Dr. Bjorn C. Martin

Dr. Charles G. Tauer

Dr. David R. Porter

Dr. A. Gordon Emslie

Dean of the Graduate College

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Chapter I

INTRODUCTION

Sorghum (*Sorghum bicolor* L.) is one of the most important cereal crops in the world in terms of both area cultivated and total yield, and is ranked fifth among the crops cultivated, following wheat, rice, maize, and barley. The haploid genome size of sorghum is 760 mega base pairs (Mb), and is smaller than the genome sizes of other crops such as wheat (16,000 Mb) and maize (2,500 Mb), but not rice (430 Mb). The chromosome number of *Sorghum bicolor* is 2n=20 (Lin et al., 1999). The greenbug (*Schizaphis graminum* Rondani) has been reported as one of the major pests of sorghum since 1968 (Porter et al., 1997), and causes tremendous economic losses in crop production to the amount of approximately $21.3 million annually in Texas alone (Katsar et al., 2002). The greenbug is not only a major pest in sorghum, but also a serious problem on many other staple crops, including wheat, where greenbug feeding causes economic losses in production to the amount of $60 to $100 million per year (Smith and Starkey, 2003). Until recently, producers have relied

mainly on insecticides for greenbug control, which can cause harmful contamination of the environment. In addition, many insecticides are costly.

The greenbug has a relatively small genome size. The genome size of greenbug is 387 Mb, and the chromosome number is 2n=8 (Ma et al., 1992). By 1997, eleven biotypes of greenbug had been reported based on differences in phenotypes, and four out of the eleven biotypes (Biotype C, D, I, and K) were reported to do harm on sorghum (Porter et al., 1997). A molecular phylogenetic analysis among the greenbug biotypes was performed based on variations in the sequence of the 1.2-kb cytochrome oxidase I gene. Sequence divergence among the 11 greenbug biotypes ranged from 0.08% to 6.17%, and these divergences were caused by host-adaptation on wild grasses (Shufran et al., 2000). The greenbug is a light greenish-yellow aphid with narrow dark green streaks down the center of the abdomen, and greenbug strains that attack sorghum differ from other aphid strains by their ability to reproduce at high temperature. The greenbug is the largest group of phloem feeding insects, and takes up photoassimilates from sieve elements in plants with its stylet mouthpart. The greenbug penetrates epidermal and mesophyll cells in plants, and probes intercellularly with a stylet mouthpart until it reaches phloem sieve elements (Dixon, 1998). In most cases, the pathway of aphid stylets is intercellular, but under certain conditions, the stylet moves toward intramural pathways within cell walls, which

causes cell wall disturbance and damage to plasma membranes of mesophyll and parenchyma cells (Moran et al., 2002). The saliva of greenbugs contains non-enzymatic reducing compounds, oxidases, and enzymes depolymerizing polysaccharides. The greenbug saliva is known to containdiverse enzymes such as pectinase, cellulase, polyphenoloxidase, peroxidase, and lipase activities (Miles, 1999). Secretion of these enzymes helps greenbugs feed more easily by lubrication of stylets, sustenance of favorable oxidation / reduction conditions, and detoxification of phenolic compounds resulting from activation of plant defense responses (Miles and Oertli, 1993).

Plants utilize diverse defense mechanisms in response to abiotic and biotic stresses efficiently by modulation of feedback and crosstalk among molecular regulators. The expression profiles of Arabidopsis produced by application of diverse treatments such as fungal infection, exposure to salicylic acid (SA), jasmonic acid (JA), or ethylene (ET) shared a substantial level of expression of the common defense genes (Schenk et al., 2000). Silencing the expression of tobacco phenylalanine ammonia lyase-encoding gene (*PAL*) weakened resistance to TMV infection, but strengthened resistance to insect feeding in tobacco. Overexpression of the *PAL* gene in tobacco was resulted in reversing the phenotype, which showed higher resistance to TMV infection and lower resistance to insect feeding (Felton et al., 1999). Among the

41 JA-responsive genes in Arabidopsis, three genes were verified to be induced via alternate signaling pathways known to be regulated by ET, auxin, and SA (Sasaki et al., 2001). Signaling cascades known to be orchestrated by JA, SA, and ET communicate with each other in synergistic or antagonistic ways against diverse biotic- and abiotic-stresses (Turner et al., 2002). A gain-of-function transgenic tobacco plant showing over-production of ET showed a unique pathway for its elicitation of plant defense responses, separate from elicitation of defense events by activation of jasmonate or methyl jasmonate biosynthesis (Kim et al., 2003). The unique pathway for ET implies existence of alternate pathways in addition to common pathways for induction of defense responses in plants. It is known that emergence of new greenbug biotypes is attributed to broad genetic variability stacked within greenbugs obtained by adaptation on diverse wild grasses during feeding (Porter et al., 1997). From these results, we can infer that insects have their own defense machineries evolved to avoid the induction of plant defense responses.

It has been reported that an array of genes is activated to defend against insect feeding and subsequent damage (Ryan, 2000). Many reports have focused on chewing damage in plants. Insect feeding by chewing and devouring plant tissues elicits common defense systems in plants, which is regulated by the well-known molecular regulator, JA. The messenger molecule, 18-amino acid polypeptide systemin, is

released in damaged tissues following mechanical wounding or insect feeding. Systemin triggers mitogen-activated protein kinase (MAPK) activation, leading to activation of the octadecanoid pathway via release of phospholipase A_2 (Stotz et al., 1999). Systemin also induces accumulation of the second messenger, hydrogen peroxide (H_2O_2), which also promotes biosynthesis of JA, leading to induction of defense genes against wounding (Orozco-Cardenas et al., 2001). JA and SA are known as universal regulators for induction of defense genes against insect feeding in plants. Low molecular mass regulators such as JA, SA, ET, and possibly H_2O_2 can modulate the expression of defense genes against diverse stresses, including pathogenesis, temperature stress, water stress, and insect feeding, by crosstalk among them (Reymond and Farmer, 1998). JA and methyl jasmonic acid (MeJA) are known as strong inducers of proteinase inhibitors, which play a pivotal role in defense responses against insect feeding.

Plants can recognize differences between mechanical wounding and insect chewing damage. Mechanical wounding generally causes a severe water stress. On the other hand, insect feeding by larvae of the cabbage butterfly (*Pieris rapae*) minimizes the water stress in Arabidopsis by avoiding damage on midveins of leaves, thereby reducing the expression of defense genes elicited by water stress (Reymond et al., 2000). A collection of 27 cDNAs in response to chewing herbivory by the tobacco

hornworm *Manduca sexta* was obtained from tobacco using differential display reverse transcription (DDRT), and the further analysis based on the cDNAs revealed that the genes involved in photosynthesis were significantly down regulated in contrast to strong up regulation of genes related to defense responses (Hermsmeier et al., 2001; Hui et al., 2003). A microarray analysis confirmed a relationship between elicitation of plant defense response and insect regurgitants/oral secretions, including fatty acid-amino acid conjugates (FACs).

Many studies have focused on plant defense mechanisms against chewing insect-feeding, but much less focus has been on plant defense responses against insect phloem-feeding, including phloem-feeding by greenbugs and white flies. Phloem-feeding produces minor injury, compared to damage elicited by chewing insects. Thus, wounds produced by insect phloem-feeding are perceived as similar to pathogen attacks in plants (Walling, 2000). Unlike chewing insects, greenbugs uptake photoassimilates by insertion of their stylet mouthparts into the phloem of host plants, resulting in a different type of damage, compared to wounds produced by chewing insects. A phloem-feeder, white fly, showed a unique expression pattern of a set of defense genes in tomato (Van de Ven et al., 2000). White flies did not induce the genes known to be involved in wounding, which are mainly induced via the octadecanoid pathway. Rather, white flies induced the genes regulated by diverse

molecular regulators such as SA, JA, and ET. Induction of plant defense genes is highly dependent on the levels of tissue damage at feeding sites. A leucine-rich repeat protein-encoding gene (*CALLRR1*) was induced in pepper by a citrus pathogen *Xanthomonas*, caused by little injury to the phloem during pathogenesis (Jung et al., 2004). In addition to direct damage inflicted by greenbugs, virus infection is sometimes accompanied with greenbug feeding. Cauliflower mosaic virus (CaMV) and barley yellow dwarf luteovirus (BYDV) are known to be introduced to plants during greenbug feeding (Peiffer et al., 1997; Palacios et al., 2002).

Evaluation of sorghum genes conferring resistance to greenbugs at the chromosomal level was performed using restriction fragment-length polymorphism (RFLP), and revealed that at least nine loci dispersed on eight linkage groups were involved in greenbug resistance in sorghum (Katsar et al., 2002). Enzymes secreted from aphid stylets inactivate functions of plant defense molecules by combining reducing compounds in aphid saliva to the defense molecules with supportof oxidases , leading to depolymerization of the plant defense molecules (Miles, 1999). The greenbug feeding on rosette leaves in Arabidopsis induces the expression of genes identified to be induced by SA and JA /ET dependent signal pathways (Moran and Thompson, 2001). The expression profiles of Arabidopsis infested with greenbugs shared commonalities with those obtained by mechanical wounding and insect

chewing damage (Moran et al., 2002). On four sorghum lines showing different resistance to aphids, fungal infection, and mechanical wounding, the expression patterns and active location of enzymatic activity of chitinase (CHI) and β-1,3-glucanase (BGL) were investigated (Krishnaveni et al., 1999). Both susceptible and resistant lines showed intense induction of both genes, but duration and cellular location of each enzyme differed with the levels of resistance and types of stress employed.

In this study, identification of expression profiles of sorghum (*Sorghum bicolor* L.) in response to the greenbug (Biotype I) was performed to pursue a better understanding of defense mechanisms against greenbug feeding. In addition, among the gene profiles, two genes, *Xa1* and *OSBP*, were further characterized and their regulation mechanisms were investigated. To produce expression profiles, two molecular biological methods, suppression subtractive hybridization (SSH) (Diatchenko et al., 1996) and microarray analysis, were used. SSH is a desirable tool for collection of differentially regulated genes in response to certain treatments by normalization and selective amplification of transcripts expressed differentially. The microarray analysis is a powerful method enabling us to investigate genome-scaled studies of gene expression in response to any desired treatment. The history of microarray technology began when Southern blotting was introduced 25 years ago. A

breakthrough of modern microarray technology came about through two crucial innovations; use of solid supports such as glass or silicone chip, and development of methods for high density oligonucleotide synthesis directly on microarray slides, including biochips. The main obstacle in the modern microarray was not from microarray itself, but from the complexity of analysis of data gathered from microarray experiments. With rapid development of computer and communication technologies, the microarray technology began to exert its full potential (Bassett et al., 1999). An application of microarray analysis along with RNA gel blot analysis is essential for high accuracy of gene profiling, as well as use of multiple replicates for microarray analysis (Rabbani et al., 2003). In this study, characterization of *Xa1* and *OSBP* genes was also performed based on the nucleotide sequences of both genes. Using several on-line programs such as ClustalW, ProtParam, and Translate, sequence analyses of both genes were performed to elucidate the structures of genes and their deduced proteins. The expression analyses of *Xa1* and *OSBP* using northern-blot analysis were performed by comparison of expression patterns of each gene in response to three different treatments; 1) greenbug infestation, 2) mechanical wounding, and 3) methyl jasmonate treatment.

RATIONALE

The aphid greenbug is a notorious pest of important crops, including wheat and sorghum. To minimize damage caused by greenbug feeding, diverse attempts of producing greenbug-resistance cultivars have been made so far, resulting in progress of development of newly resistant cultivars. Nevertheless, new greenbug biotypes have emerged periodically, making it more difficult to prevent greenbug damage. Therefore, more powerful and direct approaches to prevent greenbug damage are needed.

This study is designed to elucidate molecular interactions between sorghum and greenbug phloem-feeding. Using diverse molecular experimental methods, including SSH, microarray analysis, northern blotting, and bioinformatics, we identified sorghum genes responsive to greenbug feeding. These results are crucial in order to understand sorghum defense mechanisms against greenbugs. In collaboration with other efforts to prevent greenbug damage, this study will contribute to our knowledge of plant defense responses by expanding our understanding of molecular interactions between plants and greenbugs. Ultimately, this study may result in developing more stable and stronger greenbug resistance sorghum cultivars. Sorghum transformation mediated by particle bombardment and *Agrobacterium* infection, which contains the super-binary vector expressing the reporter gene has been successfully demonstrated

(Casas et al., 1993; Zhao et al., 2000). Successful transformation by molecular genetic engineering paves the way to introduce desired genes directly into the target plants.

OBJECTIVES

In this study, we identified the expression profiles of sorghum genes in response to greenbug phloem-feeding for a better understanding of molecular defense mechanisms of sorghum against greenbugs. Previous studies revealed that plants respond to an individual stress in a unique fashion. Therefore, it is reasonable to infer that plants will show a unique regulation pattern of genes in response to greenbug phloem-feeding. Using cDNA subtraction, microarray analysis, database search, and northern blot analysis, a total of 157 genes verified to respond to greenbug feeding were identified. Of these 157 genes, two genes, one encoding Xa1 (*Xa1*) and the other encoding oxysterol binding protein (*OSBP*), which have never been reported for their involvement in defense responses against greenbug feeding, were further characterized using sequence analysis and northern blot analysis. We expect that our results will provide a better understanding of sorghum defense mechanisms against greenbugs and subsequently help develop stable and strong sorghum cultivars resistant to greenbug feeding.

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CHAPTER Ⅱ

IDENTIFICATION OF EXPRESSION PROFILES OF SORGHUM GENES IN RESPONSE TO GREENBUG PHLOEM-FEEDING USING cDNA SUBTRACTION AND MICAROARRAY ANALYSIS

ABSTRACT

The phloem-feeding by greenbug (*Schizaphis graminum*) elicits unique interactions with their host plants. To investigate expression profiles of sorghum genes responsive to greenbug feeding, two subtractive cDNA libraries were constructed through different combinatorial subtractions in strong greenbug resistance sorghum M627 line and susceptible Tx7000 line with or without greenbug infestation. A total of 3,508 cDNAs were selected from the two cDNA libraries, and subsequent cDNA microarray and northern blot analyses were performed for identification of sorghum defense genes. In total, 157 sorghum transcripts were identified to be differentially expressed in response to greenbug feeding. The greenbug responsive genes were

classified into nine categories according to functional roles in plant metabolic pathways such as direct defense, signal transduction, cell wall fortification, oxidative burst/stress, photosynthesis, development, cell maintenance, abiotic stress, and unknown function. Overall, the profiles of sorghum genes responsive to greenbug phloem-feeding shared common identities with other expression profiles known to be elicited by diverse stresses, including pathogenesis, abiotic stress, and wounding. In addition to well-known defense related regulators such as salicylic acid, jasmonic acid, and abscisic acid, auxin and gibberellic acid were also involved in mediation of the defense responses against greenbug phloem-feeding in sorghum.

INTRODUCTION

The aphid greenbug, *Schizaphis graminum* (Rondani), has been reported as one of the serious threats in staple crops, including sorghum (*Sorghum bicolor*) (Stone et al., 2000). Greenbug damage causes tremendous economic losses in sorghum production to the amount of approximately $21.3 million annually in Texas alone (Katsar et al., 2002). The greenbug is a typical phloem-feeder, which withdraws photoassimilates and other liquid substances mainly from phloem sieve elements, as well as from xylem and parenchyma cells in plants (Klingauf, 1987). The greenbug penetrates epidermal- and mesophyll cells in plant tissues with a stylet on the mouth part, and

probes intercellularly until the stylet reaches phloem sieve elements to avoid cellular damage and minimize consequent elicitation of plant defense responses (Dixon, 1998; Walling, 2000). In addition to the immediate damage by greenbug herbivory, greenbug mediates virus spread to plants during feeding. Aphids transmit more than 275 viruses in a non-persistent manner via salivation during intercellular phloem-feeding (Powell, 2005). The greenbug belongs to Aphididae species, and it causes little perceptible damage to its host plants. Surprisingly, components in Aphididae salivary enzymes show a compositional similarity to those produced in host plants (Miles, 1999). A detailed understanding of molecular defense mechanisms against aphid phloem-feeding in sorghum will help to develop durably resistant sorghum cultivars against aphids.

Due to their sessility, plants cannot avoid surrounding threats actively. Instead, plants operate elaborate defense systems against diverse biotic and abiotic stresses by orchestration of signal pathways, leading to activation of versatile defense responses. The crosstalk between signal pathways elicited by molecular regulators in plants has been widely reported. To defend against numerous types of challenges, plants develop efficacious defense systems via the crosstalk amongst endogenous signal molecules such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET), nitric oxide (NO), and reactive oxygen species (ROS) (Reymond and Farmer, 1998). For

instance, an antagonistic relationship was observed between SA dependent resistance on pathogenesis and JA dependent resistance on insect feeding in tobacco plants (Schenk et al., 2000). In several studies, SA suppressed JA and ET dependent signal pathways and vice versa (Dmitriev, 2003). Analysis of promoter sequence regions in cytochrome P450 genes, which responded to either biotic-, abiotic stress, or both stresses, verified that the promoter regions contain common regulatory motifs (Narusaka et al., 2004).

Compared to extensive progress in understanding the molecular biology of plant defense mechanisms in response to pathogen attack, molecular interpretation of plant responses against insect feeding is much less clear (Kessler and Baldwin, 2002). The plant defense responses against insect feeding are known to be controlled by multiple molecular regulators, including JA, SA, ET, and ROS (Walling, 2000). SA plays a crucial role in expression of defense genes responding to pathogen attack (Hammond-Kosack and Jones, 1996). Accumulation of SA in plants elicits local hypersensitive responses (HR) and systemic acquired resistance (SAR) (Maleck and Dietrich, 1999). JA is known to conduct direct defense responses, including synthesis of toxic compounds, against herbivores in plants (Stotz et al., 1999; Turner et al., 2002). Ryan (2000) found that systemin released from wound sites by insect feeding invoked elicitation of signal cascades for production of JA via the octadecanoid

pathway. ET plays a pivotal role in plant development and growth (Ecker, 1995). Inhibition of ET biosynthesis resulted in significant reduction (<30%) of JA accumulation in wound sites (Wang et al., 2002). JA and ET showed a synergistic relationship in production of proteinase inhibitors and defensins in Arabidopsis (Penninckx et al., 1998). The crosstalk between molecular regulators is a complex process that shows versatile correlations. Silencing the expression of tobacco phenylalanine ammonia lyase-encoding gene (*PAL*) catalyzing an initial step of phenylpropanoid biosynthesis weakened accumulation of endogenous SA in concurrence with increment of JA biosynthesis (Felton et al., 1999). SA inhibited enzymatic action of 13*S*-hydroperoxide dehydrogenase, leading to blockage of conversion from 13*S*-hydroperoxylinolenic acid to 12-oxo-phytodienoic acid (OPDA), which is a precursor of JA biosynthesis (Pena-Cortes et al., 1993). Inhibition of proteinase inhibitors elicited by JA and methyl-JA (MeJA) resulted from SA and acetyl-SA treatments (Doares et al., 1995). During insect feeding, ROS is produced and plays an important role in signaling, by acting as an intercellular messenger (Reymond and Farmer, 1998; Walling, 2000). Activation of NADPH oxidase by wounding results in mass production of ROS, including hydrogen peroxide, and hydrogen peroxide accumulation induces biosynthesis of JA, leading to induction of the expression of defense genes against insect feeding (Orozco-Cardenas et al., 2001;

Turner et al., 2002). Inoculation of avirulent *Pseudomonas syringae* on Arabidopsis leaves elicited ROS accumulation in tissues, which were remote from the inoculated tissues, and this oxidative burst mediated systemic resistance to pathogenesis (Alvarez et al., 1998). Plants utilize blends of volatiles comprising terpenes and fatty acid derivatives in response to insect feeding (Pichersky and Gershenzon, 2002). The volatiles serve as deterrent molecules to herbivores, attractants to natural enemies of herbivores, and messengers to neighboring plants (Pare and Tumlinson, 1999).

Aphids occupy about half of insects harmful to cultivated crops (Shufran et al., 2000). Nevertheless, little is known about the molecular responses to aphid phloem-feeding in plants. Unlike chewing herbivory that produces extensive damage to plant tissues, aphids cause minor injury while feeding. Therefore, plants recognize greenbug feeding as pathogenic infection and sequential defense responses are enforced via signal cascades elicited by SA, JA, and ET (Walling, 2000). In Arabidopsis, an analysis of expression profiling in response to aphid phloem-feeding suggested that arrays of genes induced by oxidative stress, calcium-dependent signals, and pathogenesis were prevalent in the profiles (Moran et al., 2002). It has been known that plant defense responses against insect feeding are not only induced by tissue damages but also by insect saliva and regurgitants (Miles, 1999; Halitschke et al., 2001). The relationship between duration of aphid salivation and host-

susceptibility was investigated and revealed that longer aphid salivation occurred on more resistant plants, indicating high correlation between aphid salivation and evasion from plant defense responses (Ramirez and Niemeyer, 1999). The saliva of greenbugs contains non-enzymatic reducing compounds, lipase, oxidases, and enzymes depolymerizing polysaccharides such as pectinase and cellulase (Miles, 1999). The secretion of greenbug saliva may help greenbug feeding by several factors such as lubrication of the stylet, maintenance of preferable redox states, and detoxification of phenolic compounds produced by plant defense responses (Miles and Oertli, 1993). Three genes, *SLW1, SLW2*, and *SLW3* were identified to respond to whitefly-feeding in squash. The *SLW1* encoding a metallopeptidase-like protein showed up-regulated expression to exogenous MeJA and ET treatment (van de Ven et al., 2000). Zhu-Salzman et al. (2004) demonstrated that greenbug feeding on sorghum activated JA- and SA-regulated genes, likely linked to host defense responses. Normal allocation of carbon and nitrogen in alfalfa was disrupted by aphid feeding and subsequent morphological modifications followed (Girousse et al., 2005). Expression profiling of sorghum genes associated with treatments by MeJA, SA, and aminocyclopropane carboxylic acid demonstrated that both synergistic and antagonistic effects appeared in the expression ofgenes induced by SA or MeJA (Salzman et al., 2005).

Our present study pursued further understanding of sorghum molecular

defense mechanisms in response to greenbug phloem-feeding. Using two different sorghum lines, M627 (Resistant) and Tx7000 (Susceptible), twosubtract ive cDNA libraries were constructed. Subsequent cDNA microarray analyses based on the subtracted cDNA clones followed. Then, northern-blot analyses were employed to confirm data obtained from the microarray analyses. Sorghum genes that showed differential expression levels in response to greenbug feeding were identified by database searches, and then classified into functional categories. The results of this study suggest that the defense responses against greenbug phloem-feeding in sorghum are coordinately modulated by versatile molecular regulators such as SA, JA, ROS, ABA, GA and auxin. It is also suggested that greenbug phloem-feeding accompanies multiplex stresses similar to wounding, drought, oxidative stress, pathogenesis, water stress, and insect herbivory.

MATERIALS AND METHODS

Plant growth and aphid culture conditions

Seeds from the two different sorghum (*Sorghum bicolor*) lines (M627 and Tx7000) were planted (25 seeds per pot) on potting compost soil in plastic pots with

transparent plastic cages (6 inch diameter and 5.5 inch depth). The sorghum M627 line is a strong greenbug resistance line (http://www.dowagro.com/ mycogen/sorghum /grain.htm). On the other hand, the sorghum Tx7000 line has high susceptibility to greenbug phloem feeding (http://esa.confex.com/esa/2001/techprogram/paper_1814 .htm). Seedlings were grown in a greenhouse for 10 days at 29℃ and 60% relative humidity in a 14 h-light/10 h-dark photoperiod. Biotype I greenbugs are known to be the most widely spread currently in the U.S. (Tuinstra et al., 2001), and were raised on susceptible young barley seedlings in a growth chamber for 11 days at 30℃ and 60% relative humidity in a 14 h-light/10 h-dark photoperiod.

Aphid infestation on plants

For infestation, greenbugs were placed on sorghum seedlings (10-day-old) with a paint brush. To maintain heavy infestation, approximately 30 greenbugs were placed on each seedling. Greenbugs were removed at 12, 24, and 72 h after greenbug introduction by gentle tapping and air brushing. Tissues of sorghum seedlings above the soil were collected, and then frozen immediately in liquid nitrogen and stored at −80℃ prior to use.

Construction of subtractive cDNA libraries

Total RNA was extracted from 72 h greenbug-infested sorghum seedlings of M627, Tx7000, and non-infested M627, respectively, which were collected at the same time. Seedlings were ground into a fine powder in liquid nitrogen and total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA). Then, mRNA was isolated using Poly(A)Purist kit (Ambion, Austin, TX). The cDNA subtraction was carried out using the PCR-Select cDNA subtraction kit (Clonetech, Palo Alto, CA) according to the manufacturer's recommendations. In brief, two different cDNA subtractions were carried out based on a scheme that mRNA isolated from the greenbug-infested M627 was used to produce 'tester' cDNA, and mRNA from the infested Tx7000 or non-infested M627 was used to synthesize 'driver' cDNA, respectively. Two rounds of sequential PCR amplifications were followed on the basis of normalized cDNAs for selective amplification. The resultant PCR products were cloned into the pCR2.1 TA vector (Invitrogen), and transformed into *E. coli* TOP10 cells (Invitrogen). Transformed cells were cultured in liquid LB medium (Tryptone 10g, yeast extract 5g, NaCl 10g in 1ℓ LB supplemented with 270 μM ampicillin), and further screening of transformed cells was accomplished by blue-white screening. Transformed cells were stored in liquid LB medium containing 8% glycerol.

Amplification of cDNA inserts and preparation of cDNA microarray

The subtractive cDNA inserts ligated to the vector pCR2.1 were rescued by PCR amplification using primers 5'-TCGAGCGGCCGCCCGGGCAGGT-3' (Nested 1, Invitrogen) and 5'-AGCGTGGTCGCGGCCGAGGT-3' (Nested 2R, Invitrogen). Transformed cells were lysed for direct use of DNA templates for PCR reaction. To generate burst cells, 5 μl of bacterial culture was mixed with 95 μl of distilled pure water, and then mixture was incubated at 98 ℃ for 7 min. One microliter of bursted cell templates was added to 49 μl of PCR mixture containing 0.25 mM of each nucleotide, 0.4 μM of each primer, 1 X *Taq* buffer (Applied Biosystems, Foster City, CA) and 2.5 units of *Taq* DNA polymerase (Invitrogen). PCR was performed under the condition as follows: ① 98 ℃ for 5 min; ② 95 ℃ for 1 min;③ 68 ℃ for 30 sec; ④ 72 ℃ for 30 sec; ⑤ Repeat 34 more cycles from ② to ④; ⑥ 72 ℃ for 5 min. In addition, plasmids from the Arabidopsis functional genomic consortium (AFGC) microarray control set were isolated by PCR amplification, and then purified for use as normalization controls (spike 1 and spike 3). Lysates of transformed cells were used directly as DNA templates for PCR amplifications. PCR products were inspected by agarose gel electrophoresis (data not shown). Fifty microliters of each PCR

product was mixed with 125 µl ethanol and 5 µl of 5 M NH_4OAc. This mixture was blended by gentle pippeting, and then stored at -80℃ for one hour. DNA pellets were recovered by centrifugation at 4,100 rpm (3,230 G) for 40 min. After washing with 70% ethanol, the pellets were resuspended in 12 µl distilled water. A concentration of 20X SSC (3 M NaCl, 0.3 M sodium citrate) was added to the resuspended PCR products to a final concentration of 3X SSC. Each cDNA clone was printed three times on amino-silane coated slides (Corning Incorporated, Acton, MA) at the same interval using the GeneMachines OmniGrid 100 system (Genomic solution, Ann Arbor, MI) for technical replication. After printing, the slide was rehydrated with hot vapor, and snap dried on a hot plate at 80℃. Then, the slide was baked at 80℃ overnight to immobilize the cDNAs

Preparation of probes and microarray hybridization

Microarray probes were produced from total RNA of seedlings from 72 h-greenbug-infested M627 and Tx7000, as well as from non-infested M627. One hundred micrograms of total RNA from each sample was converted to cDNA using the Array 350 hybridization kit (Genisphere, Hatfield, PA). In addition, two *in vitro* transcribed normalization controls (spike 1 and spike 3) were prepared using the Riboprobe

invitro transcription systems (Promega, Madison, WI), and 100 pg of each control was mixed to the total RNA of each sample for normalization. During reverse transcription, a capture sequence was introduced to cDNA probes to arrest Cy5 and Cy3 dyes using primers containing a capture sequence. The cDNA probes were mixed with hybridization buffer (50% formamide, 8X SSC, 1% SDS, 4% Denhardt's solution), LNA dT blocker, and nuclease free water. This mixture was transferred to the slide. A 24x60 mm cover slip (Grace Bio Lab, Bend, OR) was carefully placed on the slide without creating any bubbles, and the slide was incubated at 42℃ overnight. After the hybridization, stringent washes were followed according to the manufacturer's instructions. Each hybridization reaction was repeated twice for biological replication. Probes for the replicate hybridizations were prepared from two independently prepared plant materials.

Microarray scanning and data analysis

Microarray slides were scanned using the ScanArray Express (Perkin-Elmer, Wellesley, MA) installed with two lasers, green (543 nm) and red (633 nm), aided by the ScanArray program (Perkin-Elmer). Due to the rapid deterioration of Cy5 signal intensities by exposure to the laser, scanning parameters, including laser power and

PMT (Photo Multiplier Tube) values, were determined in a small number of modulations to normalize two channels with respect to signal intensity. Normalization of signal intensity values was performed using internal controls (Spike 1 and Spike 3) spotted on the slide by modulating laser power and PMT values until the intensity ratios of both controls were as close to 1.0 as possible in order to calibrate biased signal intensities of both channels in the beginning of the scan. Each spot was put in a circle to distinguish between "spot" and "background" and the intensity of an individual spot was subtracted from background intensity and normalized using the normalization feature of the GenePix Pro program (version 4.0) (Axon Instrument, Union City, CA). Pre-processing of the normalized microarray data was accomplished using the GenePix Auto Processor (GPAP) (http://darwin.biochem.okstate.edu/gpap). This pre-processing included: 1) removal of bad quality spots; 2) removal of data where the fluorescence signal intensities in both channels were less than the background plus two standard deviations; 3) removal of data where the signal intensities in both channels were less than 200 Relative Fluorescence Units; 4) $\log_2$ transformation of the background subtracted and normalized signal intensity median ratios.

DNA sequencing and database search

The cDNAs verified to be differentially expressed against greenbug phloem-feeding were subject to sequencing reactions. Each cDNA was sequenced as follows; Lysed cells used for the synthesis of microarray cDNA probes were used as PCR templates once again. Inserts of the cDNA clones were amplified by PCR using a set of primers, M13 forward (5'-GTAAAACGACGGCCAG-3') and M13 reverse (5'-CAGGAAACAGCTATGAC-3'). To purify the PCR products, 5 μl of PCR products were mix with 0.4 μl of enzyme mix (0.5 U/ μl of each shrimp alkaline phosphatase and exonuclease I) and then incubated at 37℃ for 30 min and 85℃ for 15 min. Two microliter of each purified PCR product was added to the mixture as follows; 1 μl 5X sequencing buffer (400 mM Tris, 10 mM $MgCl_2$, pH 9), 1 μl M13 forward primer (100 ng/ μl), 2 μl BigDye® Terminator (Applied Biosystems, Forster City, CA), and 4 μl of deionized water, and then PCR was performed as follows; ① 95℃ for 30 sec, ② 96℃ for 10 sec, ③ 50℃ for 5 sec, ④ 60℃ for 4 min, ⑤ Repeat from step ② to ④ for 35 cycles. Then, PCR products were purified using Gel Filtration Cartridges (Edge BioSystem, Gaithersburg, MD). The resultant PCR products were sequenced using the ABI Model 3700 DNA Analyzer (Applied BioSystem). The database search was performed on the basis of the cDNA sequences using BLASTX and BLASTN. BLASTN was used in case of absence of any matched hits when performing

BLASTX. All cDNA sequences were submitted to the GenBank dbEST, and accession numbers are listed in Table 2-1.

Northern-blot analysis

Total RNA was isolated from seedlings collected after three different time points of greenbug infestation (12, 24, and 72 h), as well as from non-treated control sorghum materials (10-day-old) in the same manner as above. Approximately 10 μg of total RNA per sample was fractionated in a 1% agarose gel containing 1.1 M formaldehyde, and then transferred to Hybond-N^+ membrane (Amersham Biosciences, Piscataway, NJ) using the alkaline solution (3 M NaCl and 0.01 N NaOH) transfer method. Probes were labeled with ^{32}P-dCTP (Perkin-Elmer) using PCR amplification of cDNA inserts from the pCR2.1 vector and hybridized to the membrane soaked with 2ml of the UltraHyb buffer (Ambion) at 42℃ overnight. Then, the hybridized blots were washed with 2X SSC/ 0.1% SDS at 65℃ and 0.1X SSC/ 0.1% SDS at 60℃ and exposed on Kodak BioMax MS film (Kodak, Rochester, NY) at -80℃ overnight.

RESULTS

Expression profiling of sorghum genes responsive to greenbug phloem feeding

In this study, two different sorghum lines known to possess different levels of greenbug resistance were used to profile greenbug responsive genes for a better understanding of sorghum defense mechanisms against greenbug feeding. Seedlings of the sorghum M627 line showed few necrotic spots and maintained a healthy green color after 72 h of greenbug-infestation, but those of the Tx7000 line exhibited widespread necrotic spots and severe wilting under the same treatment (Fig. 2-1, b and c). Two subtractive cDNA libraries enriched in genes responsive to greenbug feeding were constructed from the sorghum lines, M627 and Tx7000. A collection of 3,508 cDNA clones were obtained from the cDNA libraries and printed on specially designed glass slides for the microarray analyses.

Based on the collected cDNAs, two microarray analyses were performed. Each microarray analysis was designed to investigate expression patterns of transcriptome profiles from two different combinations of sorghum plants, greenbug infested M627 (Mi) versus non-greenbug infested M627 (Mni) and Mi versus greenbug infested Tx7000 (Ti). In the microarray analyses, expression profiles of sorghum genes showing induction or suppression in response to greenbug feeding were investigated. To increase reliability and consistency of the microarray analyses,

application of multiple replicates was adopted following the suggestion from Ting Lee et al. (2000). To perform each microarray analysis, two independently prepared biological replicates and three technical replicates were used to minimize variability of results. To avoid technical bias of intensity ratios between Cy5- and Cy3 fluors, the intensity ratio of each clone was normalized using two normalization control features (Spike 1 and Spike 3) synthesized from two human genes encoding B-cell receptor-associated protein and myosin light chain 2, respectively, and spotted on the slide. In addition, the significance of correlations in expression fold changes among the replicates of each cDNA was considered by statistical analyses provided in the GPAP. In this study, genes were considered to be differentially regulated if intensity ratios of cDNA clones from the microarray analyses showed more than a 1.8-fold change of expression up or down. Two scatter plots representing distribution of signal intensity patterns of cDNAs printed on the slide for the microarray analyses are shown (Fig. 2-2, a and b). On average, approximately 18% (651/3,508) of the transcripts were found to be up- or down regulated more than 1.8-fold by greenbug feeding in the microarray analyses. In total, we obtained 157 genes that showed greater than a 1.8-fold induction or suppression after removal of redundant transcripts and statistically non-significant data. It is believed that these genes are involved directly or indirectly in sorghum defense responses against greenbug attack.

Co-regulation patterns of greenbug responsive genes

In the two different microarray analyses, some genes responsive to greenbug feeding were found to be co-regulated in both microarray analyses. The microarray analyses showed 72 upregulated genes in comparison of Mi to Mni, and 82 up-regulated genes in Mi-Ti comparison. Among the upregulated genes, 11 genes were commonly upregulated in both microarray analyses (Fig. 2-3a). The 11 genes commonly up-regulated belong to various functional categories such as cell wall fortification, defense, signal transduction, oxidative burst/stress, development, cell maintenance, and unknown function. On the other hand, 12 genes were suppressed in the microarray analysis between Mi and Mni, and 42 genes were down regulated in the microarray analysis between Mi and Ti in response to greenbug feeding. Out of a total of 54 down regulated genes, two genes encoding catalase and WD domain G-beta repeat containing protein were commonly down-regulated in both microarray analyses (Fig. 2-3b).

Functional classification of genes

A total of 157 genes differentially regulated in response to greenbug feeding are listed and categorized according to the putative function of each gene (Table 2-1). The signal intensity ratios of these genes from the two microarray analyses are also provided in Table 2-1. The putative functions of these genes were inferred from metabolic processes known to be related to each gene. Even though some genes were involved in multiple metabolic processes, they were classified according to their main roles in plant metabolism. The sorghum genes responsive to greenbug feeding were classified into nine functional categories such as direct defense, signal transduction, cell wall fortification, oxidative burst/stress, photosynthesis, development, cell maintenance, abiotic stress, and unknown function. The genes with unknown function occupy the largest category, and the group of signal transduction genes was ranked the second largest group, followed by cell maintenance (Fig. 2-4).

Defense-related genes

A group of genes involved in biosynthesis of defense molecules was either up- or down regulated by greenbug feeding (Table 2-1). In total, 18 genes involved in direct defense responses were differentially expressed in both microarray experiments. These genes encode well-known defense molecules, including cysteine proteinase inhibitors (CPIs), polyphenol oxidase, legumain, glucosidase, thionin, glucanase, cysteine proteinase and S-like RNase. A gene encoding CPI, a well-known plant

defense molecule against insect herbivory (Botella et al., 1996), was induced during the earlier stage (12 h) of greenbug infestation (Fig. 2-5) and maintained a high level of induction until 72 h post-infestation. Polyphenol oxidase (PPO) catalyzes biosynthesis of active quinones which are toxic to herbivores and pathogens due to their ability to produce indigestible modified amino acids and proteins (Li and Steffens, 2002). The *PPO* gene was induced from 72 h of greenbug infestation (Fig. 2-5). Thionin is a cysteine-rich antimicrobial protein induced by infection of fungi and bacteria (Oh et al., 1999). Intense induction of the thionin gene (*Thi*) was observed from 12 h to 72 h of greenbug infestation (Fig. 2-5). The genes encoding Xa1 protein (*Xa1*) and cytochrome P450 protein (*CYP*) were co-upregulated in both microarray analyses. Xa1 is a bacterial blight-resistance protein and known to confer resistance against pathogen attack by recognizing pathogen-related particles and eliciting defense responses in the cytosol (Yoshimura et al., 1998). The expression of the *Xa1* gene was induced from 72 h of greenbug infestation, after having been suppressed at 12 h and 24 h (Fig. 2-5). The cytochrome P450 enzyme is known to play multiple roles, including biosynthesis of defense compounds such as camalexin and dhurrin (Zhou et al., 1999; Bak et al., 2000). The gene encoding cysteine proteinase (*CP*) was induced from 72 h of greenbug infestation (Fig. 2-5). Pechan et al. (2000) demonstrated that the *CP* gene was induced by larval feeding, and CP participated in inhibition of lepidopteran larvae growth in maize.

Cell wall fortification

Nine genes involved in cell wall fortification were up- or down regulated by greenbug

infestation (Table 2-1). The genes encoding caffeic acid *O*-methyltransferase (*COMT*) and proline-rich protein (*PRP*) were co-upregulated in both microarray analyses. COMT participates in lignification of cell walls (Nikolaeva, 2000; Morreel et al., 2004), and PRP is known to be a structural component of cell walls, and involved in cell wall reinforcement (Vignols et al., 1999). The *COMT* gene was induced after 72 h of greenbug infestation in both microarray analyses (Fig. 2-5, Fig. 2-6), and the *PRP* gene was upregulated at 12 h after greenbug infestation (Fig. 2-6).

Signal transduction

In total, 26 genes involved in signal transduction were expressed differentially in response to greenbug feeding (Table 2-1). The number of genes in this category makes up the second largest category, next to the category of unknown function. Among these genes, a gene-encoding Ras-GTPase activating protein binding protein (*Ras*) was significantly up- or down regulated. The Ras-GTPase is known to play a crucial role in controlling mitogen-activated protein kinases (MAPKs) and transduces diverse signals in animals (Shields et al., 2000). In Arabidopsis, Ras-GTPase is absent and the role of Ras-GTPase is carried out by Rop-GTPase (Li et al., 2001). The expression of *Ras* showed reverse patterns between the two microarray experiments.

In the microarray analysis between Mi and Mni, the *Ras* gene was induced from 72 h of greenbug infestation, but suppressed in the analysis between Mi and Ti from 12 h of greenbug infestation. This suppression of *Ras* resulted from higher upregulation of *Ras* in Ti than in Mi at 72 h of the infestation (Fig. 2-5, Fig. 2-6). A gene encoding ankyrin-induced protein was upregulated. Ankyrin regulates the SA-dependent defense reactions, including systemic acquired resistance (Cao et al., 1997; Lu et al., 2003).

Oxidative burst/stress involved genes

The genes encoding peroxidase (*PX*), gluthathion-*S*-transferase (*GST*), catalase (*CAT*), and quinone oxidoreductase (*QR*) were up- or down regulated by greenbug feeding (Table 2-1). Both PX and CAT play a key role in controlling ROS concentration, leading to oxidative signal transductions (Kawano, 2003). The *CAT* gene was suppressed from 12 h of greenbug infestation, but the *PX* gene was induced from 12 h of greenbug infestation and reached a peak point at the 24 h time point (Fig. 2-5). QR scavenges toxic free radical semiquinones using divalent reduction, and was induced by oxidative stress in Arabidopsis (Mano et al., 2002).

Abiotic stress involved genes

Four genes encoding starch synthase (*SS*), heat shock protein (*Hsp*), phytochelatin synthetase (*PCS*), and ABA-water stress-ripening-induced protein (*ASR*) showed differential regulation in response to greenbugs. The genes encoding starch synthase (*SS*) and heat shock protein (*Hsp*) were reported to participate in plant theromotolerance and protection of electron transport in photosystem II (Heckathorn et al., 1998; Majoul et al., 2004). Upregulation of the *SS* gene was reported on wheat under heat stress (Majoul et al., 2004), and rapid changes in expression of the *SS* gene were also reported in water-stressed wheat plants to control photoassimilation (Ahmadi and Baker, 2001). The *SS* gene was induced from 12 h of greenbug infestation, and gradually increased its induction with extension of the infestation (Fig. 2-5). Induction of the *ASR* gene for protection of plant DNA under water-stressed conditions is known to be controlled by the phytohormone ABA (Riccardi et al., 1998). Two sorghum genes, the aldehyde oxidase gene and the drought-, salt-, and low temperature responsive gene (*DRT*), which are known to be regulated by ABA, were profiled in response to greenbugs (Zhu-Salzman et al., 2004). Considering our results and previous reports, it is plausible that ABA participates in regulation of sorghum defense responses against greenbugs.

Genes involved in cell maintenance

As shown in Table 2-1, 25 genes involved in cell maintenance showed differential expression by greenbug infestation. Several genes encoding 40S- and 60S-ribosomal protein subunits were upregulated in both microarray analyses. Differential expression of genes encoding alpha- and beta-tubulin was also shown. Previous studies suggest that the diverse stresses, including water deficiency and hyperosmosis can elicit changes in composition and conformation of cell cytoskeletons consisting of tubulins (Komis et al., 2002). A gene encoding alpha tublin was upregulated by application of *Cis*-jasmone, a well-known plant hormone involved in defenses against insect herbivory (Birkett et al., 2000). An actin-encoding gene was also found to be upregulated in this study. Compositional changes of actin cytoskeletons in plant cells are involved in defense events during pathogenesis (Kobayashi and Hakuno, 2003). A gene encoding aspartate aminotransferase (*AAT*) was down regulated. AAT is known to play a pivotal role in nitrogen and carbon metabolism, especially in C_4-plants and legumes (Silvente et al., 2003), and suppression of the *AAT* gene was reported in Penjalinan plants under drought conditions (Aroca et al., 2003). The gene encoding histone H2A (*H2A*) was induced from 12 h to 24 h of greenbug infestation, and

reversed to suppression from 72 h of greenbug infestation (Fig. 2-5). Intense induction of the *H2A* gene was reported in drought stressed hot pepper plants (Park et al., 2003).

Development-related genes

A group of genes encoding auxin induced protein (*AIP*), GA induced protein (*GIP*) and seed maturation protein was either up- or down regulated by greenbug feeding. A gene encoding AIP was co-upregulated in both microarray analyses (Table 2-1). The *GIP* gene was induced from 72 h of greenbug infestation, and the *AIP* gene was also upregulated from 72 h of greenbug infestation (Fig. 2-5, Fig. 2-6). The plant hormones auxin and GA have been widely known to be involved in plant development. They also negatively affect expression of several defense genes in plants, and show antagonistic relationships with defense related hormones such as ABA and ET (Mayda et al., 2000).

Photosynthesis-related genes

A number of genes involved in photosynthesis were up- or down regulated by greenbug feeding (Table 2-1). Ferredoxin (Fd) is an iron-sulfur containing protein of

chloroplast photosystem I, and promotes harpin-mediated HR (Dayakar et al., 2003). The *Fd* gene was induced from 12 h of greenbug infestation (Fig. 2-6). Various biotic- and abiotic-stresses, including plant hopper phloem-feeding in rice, cause suppression of photosynthesis (Watanabe and Kitagawa, 2000). The JA suppresses expression of photosynthesis-related genes (Creelman and Mullet, 1997). This suppression is attributed to redistribution of energy to reinforce defense responses (Zhu-Salzman et al., 2004). Our data showed prevalent induction of photosynthesis related genes in the microarray analysis between Mi and Ti (Table 2-1). It is plausible that severe damage inflicted on seedlings of Ti by greenbug feeding caused irreversible failure of the photosynthetic machinery, leading to reduced expression of photosynthesis-related genes in Ti.

Genes of unknown function

The genes with unknown function ranked as the largest group of all nine categories (Fig. 2-4). A total of 46 cDNAs failed to match any sequence in the GenBank databases by the BLAST search, or matched sequences whose functions have not been characterized yet. Five genes of unknown function were co-upregulated, and two were verified to be antagonistically regulated in the two microarray analyses (Table 2-

1). Some of them showed strong up- or down regulation by greenbug feeding. This implies that these genes are intimately involved in regulation of sorghum defense responses against greenbugs.

DISCUSSION

In this study, two sorghum lines possessing contrasting levels of greenbug resistance were used for cDNA subtraction and microarray experiments to maximize the possibility of profiling genes responsive to greenbug feeding. In these comparative analyses with a 3.5K cDNA microarray, a total of 157 transcripts were identified to be responsive to greenbug feeding. The resultant profiles are more comprehensive than other aphid-induced gene profiles reported earlier (Moran et al., 2002; Voelckel et al., 2004; Zhu-Salzman et al., 2004). These comparative approaches not only allowed us to profile genes which were not identified in previous studies, but also to confirm the genes previously identified to be responsive to greenbug feeding. Compared to a previous study (Zhu-Salzman et al., 2004) conducted with a similar purpose, our results mostly showed consistent results, and also exhibited novel data contributing to a better understanding of plant defense responses against greenbugs. It is believed that most new findings in our study resulted from the use of two contrasting sorghum lines

showing either strong greenbug-resistance or susceptibility. Unlike previous reports by Zhu-Salzman et al. (2004) and other groups, which focused on aphid-induced responses of a susceptible host plant, this study showed differential responses against greenbugs by comparative analyses between resistant and susceptible lines. Thus, the defense responsive genes identified in the resistant source may contribute to a strong resistance to greenbugs.

Phloem-feeding aphids represent a special model in studies of plant-insect interactions. When aphids attack host plants, they penetrate plant tissues and probe intercellularly with their stylet-like mouth parts to feed on nutrients translocating via phloem-sieve elements. Once the feeding structure is formed, the aphid can continue feeding at the same site for several days. Consequently, plants may have defense systems offering both quick and long-lasting responses. Thus, it is important to select an appropriate time point to profile the genes responsive to greenbugs. Moran and Thompson (2001) showed that a majority of aphid-induced genes, including genes which induced systemic defenses, peaked at three days post-infestation (dpi) in Arabidopsis. We therefore analyzed the gene expression in sorghum plants at three dpi with greenbugs. As a consequence of the difference in sampling time and comparative analyses, the profiles obtained in this study have a wide coverage of differentially expressed genes, especially these late-responsive genes, when compared with other

profiles constructed using greenbug-induced sorghum seedlings collected at two dpi (Zhu-Salzman et al. 2004).

In our data, a portion of the responsive genes was identified to be regulated via SA- and JA-dependent signal cascades. This supports a paradigm that phloem-feeding elicits intermediary responses between wounding and pathogen infection (Moran and Thompson 2001). During phloem-feeding, aphids secrete saliva for multi purpose, including lubrication of stylets, optimization of redox conditions in plants, and prevention of plant defense responses (Miles, 1999; Moran et al., 2002). Plants have developed elaborate defense systems to confront these elusive challenges by aphids. Plants recognize components in aphid saliva that elicits reinforcement of the defense responses (Zhu-Salzman et al., 2005). In addition, plants perceive elicitors released from greenbug feeding sites, which triggers the onset of plant defense responses (Schilmiller and Howe, 2005). Binding of the elicitor systemin to the receptor SR160 activates phospholiapse, leading to release of linolenic acid, which is a precursor of JA (Ryan and Pearce, 2003). JA synthesized from linolenic acid is strongly involved in induction of defense responses against insect feeding, mechanical wounding, and pathogen attack (Seo et al., 2001). Likewise, SA controls defense signaling in response to pathogen attack in plants. SA plays a pivotal role in regulation of local- and systemic-defenses, including induction of HR and SAR, as

well as expression of pathogenesis-related (*PR*) genes (Durner et al., 1997). In our profiles, several genes elicited by SA and JA were identified to encode diverse proteins, including CPI, polyphenol oxidase, glucanase, catalase, ankyrin, cytochrome P450 monooxygenase, glutathione-S-transferase, and stearoyl-acyl carrier protein desaturase. Stearoyl-acyl carrier protein desaturase (S-ACP-DES) plays a key role in JA- and SA-dependent defense responses (Kachroo et al., 2004). S-ACP-DES converts stearic acid (18:0) to oleic acid (18:1). This conversion is a key step in maintaining the level of unsaturated fatty acids, leading to activation of JA-mediated defense responses and repression of the SA signaling cascade (Kachroo et al., 2003). The differential expression of the *S-ACP-DES* gene implies that interactions occurred between JA and SA during elicitation of sorghum defense responses against greenbug feeding.

For a deeper insight into the defense mechanisms of sorghum against greenbug feeding, two different microarray analyses were designed and performed. Unlike the first expectation, patterns of gene regulation in the two microarray analyses showed extensive dissimilarities. The dissimilarities were probably attributed to a severe difference in the level of damage inflicted on the seedlings of Mi and Ti at the time of harvesting, as well as differences in genotypes between the two sorghum lines. After 72 h of greenbug infestation, Mi maintained healthy green seedlings

nearly equal to those from untreated control sorghum (Fig. 2-1a). In addition, a portion of the greenbugs infesting Mi fell down to the ground and died for unidentified reasons. By contrast, Ti showed severe wilting and widespread necrotic spots (Fig. 2-1b, c). The microarray analysis between Mi and Mni showed overall upregulation of defense related genes in concurrence with up- and down regulation of oxidative burst related genes. The genes related to oxidative burst, encoding CAT, PX, and QR, quench H_2O_2 generation thatleads to the induction of the defense responses in plants (Orozco-Cardenas et al., 2001). The up- and down regulation patterns of the oxidative burst-related genes imply that ROS accumulation and detoxification of ROS occurred simultaneously during greenbug feeding. The microarray analysis between Mi and Ti showed overall down regulation of the *CAT, PX*, and *QR* genes with concurrent down regulation of several defense-related genes. The reason for down-regulation of defense-related genes in spite of down-regulation of oxidative burst-related genes remains uncertain, but we assume that ROS burst occurred intensely in Ti during the early stage of greenbug feeding. Therefore, levels of ROS remained high enough to induce defense-related genes before harvesting seedlings of Ti, even though scavenging of ROS has already begun. Strikingly, defense-related genes were verified to be upregulated in both Mi and Ti. For instance, our northern-blot analyses showed that the genes encoding beta-glucosidase (*Glu*) and beta glucanase (*BGL*) were much

more highly induced in Ti than the expression levels of those genes in Mi (Fig. 2-6). The question remains about what factors caused Mi to possess a strong resistant phenotype to greenbug, compared to high susceptibility of Ti. Considering the results from both microarray analyses, reinforcement of cell walls presumably played a crucial role in conferring resistance to greenbugs in M627 line.

Reinforcement of cell walls is one of the major defense strategies employed by plants (Minorsky, 2002). Two genes, *COMT* and *PRP,* were co-upregulated in both microarray analyses, and other genes involved in cell wall fortification were also upregulated, respectively. In our profiles, genes related to cell wall fortification include cellulose synthase (*Ces*), glycosyltransferase (*GT*), and pyrroline-5-carboxylate dehydrogenase (*P5CDH)*. The *Ces* was reported to be upregulated by MeJA treatment on sorghum seedlings, and differentially regulated by fungal infection (Schenk et al., 2000; Salzman et al., 2005). GT is known to play a key part in cellulose synthesis, and P5CDH is involved in the control of proline degradation (Holland et al., 2000; Deuschle et al., 2004). Strong induction of the *P5CDH* gene was observed in Ti from 12 h of greenbug infestation on the contrary to noticeably minor induction at 24 h of greenbug infestation in Mi (Fig. 2-6). This supports the idea that cell wall fortification plays a crucial part in a strongly resistant phenotype against greenbug feeding in Mi. However, a previous study (Zhu-Salzman et al.,

2004) showed the lack of cell wall fortification-related genes when using only a susceptible sorghum line challenged with greenbugs.

Here we presented the transcriptome profiles of sorghum genes in response to greenbug phloem-feeding and interpreted the regulation patterns of greenbug-responsive genes in sorghum. In addition, putative functions of genes were identified and linked to plant metabolic processes to understand mechanisms of sorghum defense systems against greenbug phloem-feeding. Some of the transcriptome profiles were verified to be controlled by several molecular regulators, including SA, JA, ABA, auxin, and GA. A gene encoding AIP, which was co-upregulated in both microarray analyses, was profiled. Two other genes encoding GA-induced protein and another auxin-regulated protein were also differentially regulated in response to greenbug feeding (Table 2-1). Precise roles of auxin and GA in defense events against greenbug phloem-feeding have remained elusive. Auxin homeostasis and maintenance of capturing auxin signaling are important in mounting defense responses (Mayda et al., 2000). GA is a well-known growth-regulator, but its role in defense events is not clear. A previous study showed that a GA treatment enhanced the germination rate of chick pea seeds, which was repressed by salt stress by increasing amylase activity and starch translocation rate (Kaur et al., 1998). Interactions between plant and insect are extremely complex, and much remains to be studied. In particular, investigation in the

field of interactions between phloem-feeding insects and plants has been little exploited and remains to be explored in spite of recent progress. More studies are required to elucidate a detailed mechanism of inducing plant defense responses by phloem-feeding insects. Additionally, more efforts on interpretation of complex interactions among molecular regulators will pave the way for understanding control mechanisms of defense events in plants.

In conclusion, using a combination of cDNA subtraction and microarray analysis, sorghum genes responsive to greenbug phloem-feeding were profiled and identified. In total, 157 transcripts verified to be involved in defense responses against greenbugs were obtained. Amongst the profiles, several genes, including *Thi* and *Xa1*, were newly identified to be involved in defense responses, directly or indirectly, on phloem-feeding herbivory. In addition, two molecular regulators, auxin and GA, were verified to be involved in the regulation of defense responses against greenbugs in sorghum. Lastly, cell wall fortification appears to be an important factor in determining assignment of resistance to greenbugs.

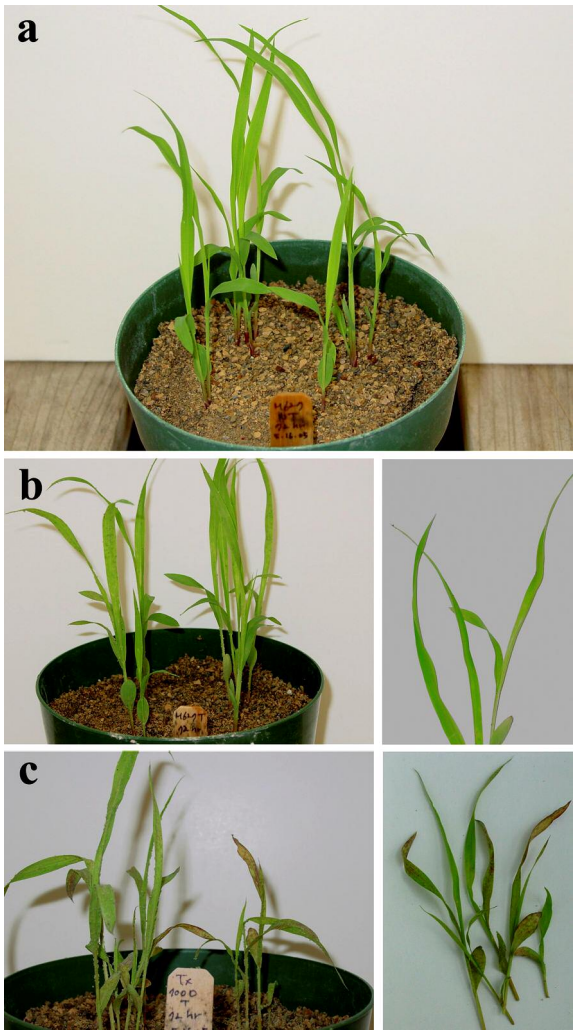

Fig. 2-1 Phenotypes of seedlings from different sorghum lines after 72 h greenbug infestation. **a**, Seedlings of sorghum line M627 with no greenbug infestation, harvested at the same time point with (b) and (c) **b**, Phenotype of M627 seedlings after 72 h greenbug infestation (left). Closer view of 72 h greenbug infested M627 seedlings (right). **c**, Phenotype of Tx7000 seedlings after 72 h greenbug infestation (left). Closer view of 72 h greenbug infested Tx7000 seedlings (right).

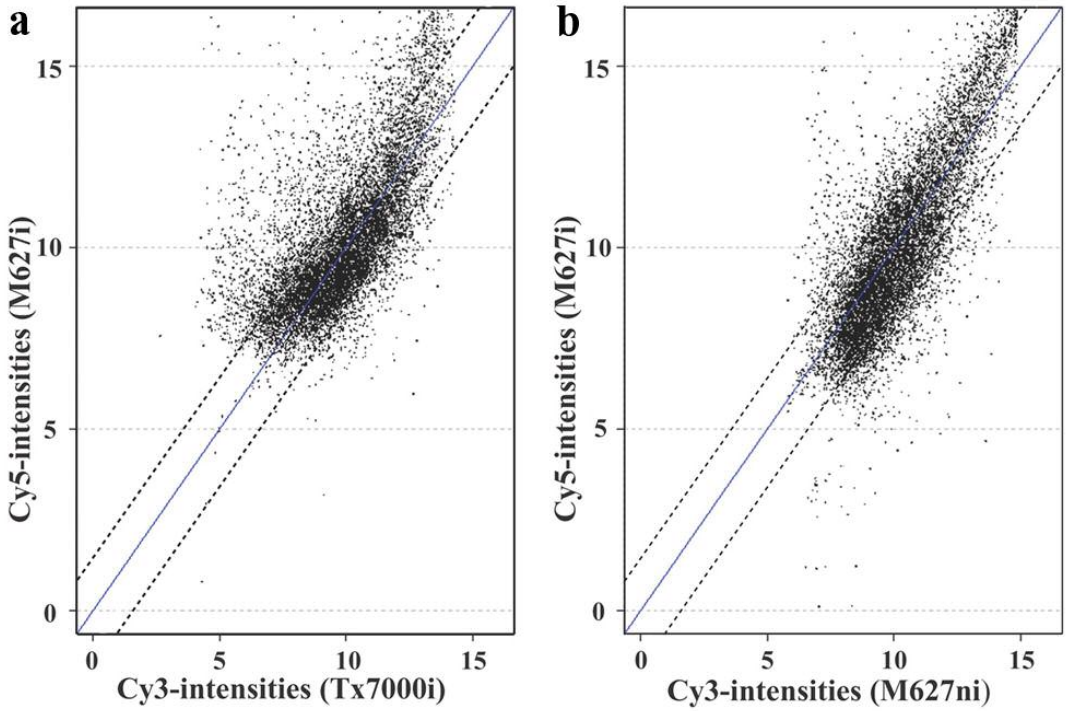

Fig. 2-2 Two scatter plots showing distribution of normalized expression patterns of cDNA clones following the microarray hybridizations. **a**, Scatter plot of normalized log 2 intensities of Cy3 (Tx7000 greenbug-infested) versus log 2 intensities of Cy5 (M627 greenbug-infested). **b**, Scatter plot of normalized log 2 intensities of Cy3 (M627 non-greenbug infested) versus log 2 intensities of Cy5 (M627 greenbug-infested). Solid line represents a 1:1 ratio of signal intensity. Dotted lines indicate 1.8-fold induction (upper-dot line) or suppression (lower-dot line) of gene expression. Normalized intensity ratios are shown for all features prior to data filtering (intensity ratios of replicates were included).

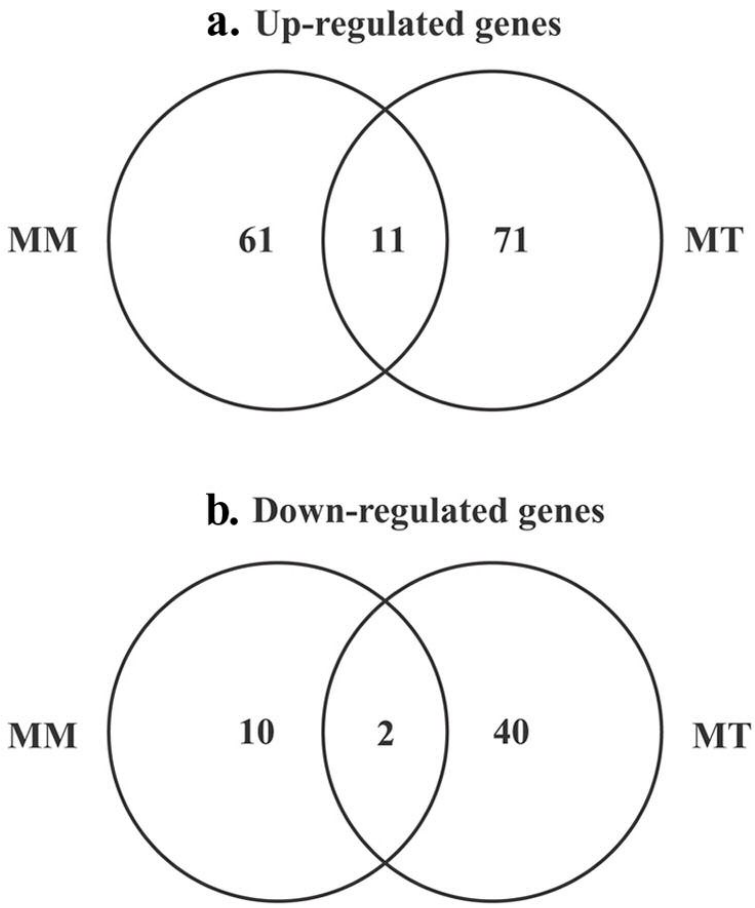

Fig. 2-3 Venn diagrams of genes differentially expressed by greenbug feeding in the two different microarray analyses. MM indicates the microarray analysis between greenbug infested M627 and non-greenbug infested M627, and MT indicates the microarray analysis between greenbug infested M627 and greenbug infested Tx7000. **a**, Numbers of genes which were induced more than 1.8-fold in MM and MT. **b**, Numbers of genes which were suppressed more than 1.8-fold in MM and MT.

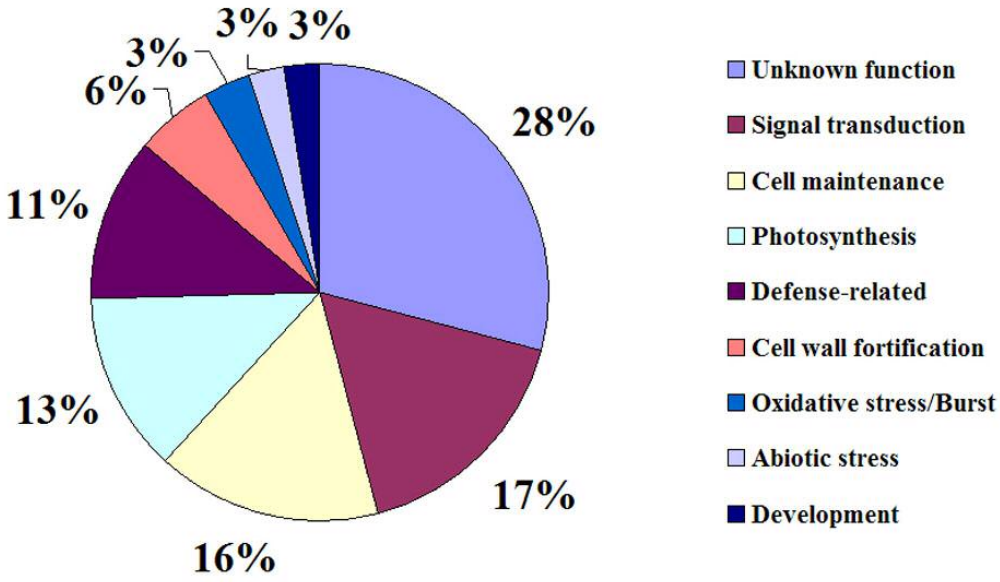

Fig. 2-4 Functional categories of the sorghum genes responsive to greenbug phloem-feeding. In pie chart, values of percentage indicate the proportion of a number of genes in each category to total number of genes (157 genes), and the functional categories were annotated (right).

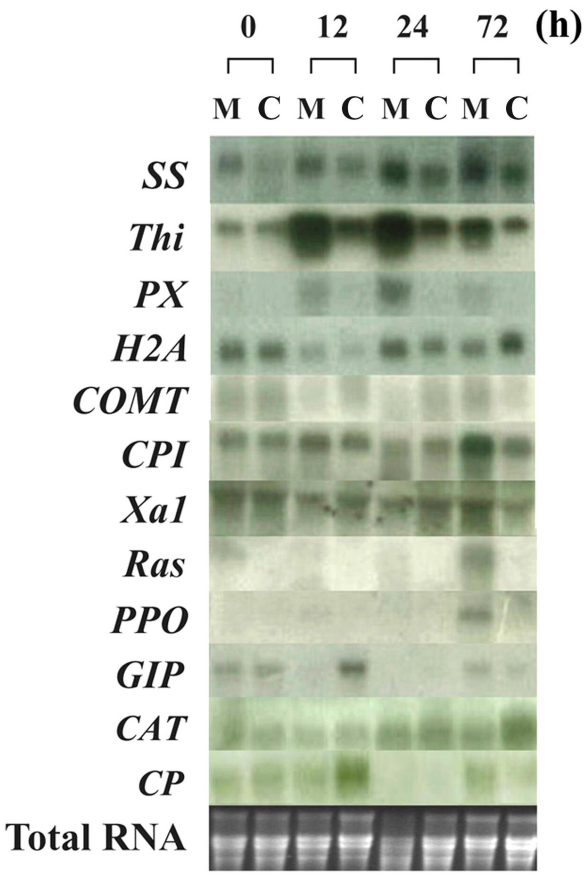

Fig. 2-5 Northern-blot confirmation of the cDNA microarray analysis. Total RNAs were extracted from greenbug-infested M627 and -uninfested M627 sorghum seedlings at 0, 12, 24, and 72 h after greenbug infestation for northern-blot analysis. Equilibrium of RNA loading was verified by intensity of total RNA bands. M, M627 greenbug infested; C, M627 untreated controls; *SS*, starch synthase; *Thi*: sulfur rich/thionin protein; *PX,* peroxidase; *H2A,* histone H2A; *COMT,* caffeic-acid *O*-methyltransferase; *CPI,* cysteine proteinase inhibitor; *Ras*, Ras GTPase activating protein binding protein; *PPO,* polyphenol oxidase; *GIP,* gibberellin induced protein; *CAT,* catalase; *CP*, cysteine proteinase.

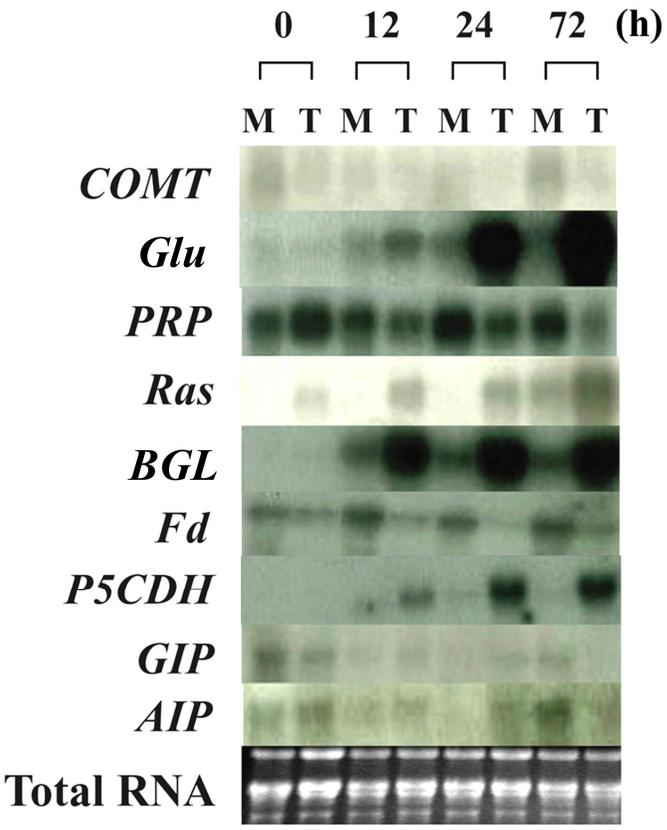

Fig. 2-6 Northern-blot confirmation of the cDNA microarray analysis. Total RNAs were extracted from greenbug infested-M627 and -Tx7000 sorghum seedlings at 0, 12, 24, and 72 h after greenbug infestation for northern-blot analysis. Equilibrium of RNA loading was verified by intensity of total RNA bands. M, M627 greenbug infested; T, Tx7000 greenbug infested; *Glu,* beta-glucosidase; *PRP*, proline rich protein; *BGL,* beta-glucanase; *Fd,* Ferredoxin; *P5CDH,* pyrroline-5-carboxylate dehydrogenase; *AIP,* auxin induced protein.

Table 2-1 *Measurement of changes in the expression of genes responsive to greenbug phloem-feeding*.

[a] BLASTX was used to determine homologous genes and putative functions of genes. BLASTN was used in case of failure to return any hits by BLASTX. [b] Values of signal intensity ratios showing up- or down regulation more than a 1.8-fold were shaded with pale blue or yellow as in order. The values of the signal intensity ratio were determined by calculating a median value of signal intensity ratios of replicates. [c] N/A indicates 'not available' due to the low significance of data. [d] GenBank accession number. All cDNA sequences were submitted to the GenBank database.

Clone	Putative function/homology/species[a]	Signal intensity ratios[b,c]		Score/e-value	Accession No.[d]
		M627i vs M627ni	*M627i vs Tx7000i*		
	Abiotic stress				
MM1	Soluble starch synthase_*Sorghum bicolor*	10.476	-16.089	120/2e-26	DR831413
MT158	Phytochelatin synthetase-like protein 1_*Sorghum bicolor*	1.238	1.796	54/2e-04	DR831443
MT32	ASR2 protein_*Oryza sativa*	2.155	-4.228	68/1e-10	DR831414
MM15	Heat shock protein70_*Oryza sativa*	3.964	-2.255	213/2e-54	DR831415
	Cell wall fortification				
MT40	Delta1 pyrroline-5-carboxylate dehydrogenase_*Oryza sativa*	1.936	-3.88	102/4e-21	DR831418
MT29	Glycosyl transferase_*Sorghum bicolor*	2.01	-3.595	52.8/3e-06	DR831419
MM108	Glycosyl transferase-like protein_*Oryza sativa*	2.043	-1.183	115/3e-25	DR831416
MT112	2-dehydro-3deoxyphosphooctonate aldolase_*Oryza sativa*	-1.319	2.439	86.3/3e-16	DR831421
MM25	Caffeic acid O-methyltransferase_*Sorghum bicolor*	3.568	2.882	64.7/8e-10	DR831420
MT89	d-TDP glucose dehydratase_*Phragmites australis*	1.022	3.069	271/1e-71	DR831422
MT80	Cellulose synthase catalytic subunit10_*Zea mays*	1.669	3.297	61.9/7e-07	DR831429
MT69	Cellulose synthase-7_*Zea mays*	-1.534	3.635	87.7/7e-15	DR831430
MM36	Proline rich protein_*Zea mays*	4.649	4.983	67/3e-10	DR831431
	Cell maintenance				
MM75	Adenine nucleotide translocator_*Zea mays*	2.637	-2.041	122/3e-27	DR831565
MT33	Aspartate aminotransferase_*Oryza sativa*	1.141	-4.09	175/4e-43	DR831432
MT179	Adenine phosphoribosyltransferase-form2_*Oryza sativa*	1.05	-2.301	178/1e-43	DR831433
MT50	RING-H2 finger protein RHG1a_*Oryza sativa*	1.003	-2.286	135/4e-31	DR831434
MM113	Actin_*Triticum aestivum*	1.796	-1.643	200/1e-50	DR831435
MM67	ATP/ADP translocase_*Zea mays*	2.572	-1.23	94.4/9e-19	DR831436
MM58	Ubiquitin ligase SINAT5_*Oryza sativa*	2.856	-1.121	113/2e-24	DR831437
MM104	ADP-glucose pyrophosphorylase small subunit_*Zea mays*	1.97	-1.056	127/1e-28	DR831566
MM93	60S ribosomal protein_*Oryza sativa*	2.151	1.07	207/1e-52	DR831438
MM110	Ribosomal protein S7_*Oryza sativa*	1.838	1.189	140/2e-32	DR831439
MM9	40S ribosomal protein_*Oryza sativa*	4.887	1.218	173/2e-42	DR831440
MM106	60S ribosomal protein L24_*Oryza sativa*	1.989	1.252	119/3e-26	DR831441
MM30	CTP synthase_*Oryza sativa*	-2.264	1.645	140/1e-32	DR831442
MT170	RNA polymerase subunit_*Oryza sativa*	-1.179	1.834	149/3e-35	DR831444
MT147	ATP-dependent transmembrane transporter_*Oryza sativa*	1.385	2.142	137/8e-32	DR831445
MM54	Alpha tubulin_*Zea mays*	2.59	2.732	150/1e-35	DR831446

Table 2-1 *Continued*

MT101	Small nuclear ribonucleoprotein polypeptideE_*Oryza sativa*	-1.115	2.811	131/6e-30	DR831447
MT95	Suppressor of actin1_*Oryza sativa*	-1.134	2.904	38.1/0.083	DR831448
MT96	Bundle sheath cell specific protein1_*Zea mays*	-1.613	3.158	106/5e-22	DR831449
MT174	NOD26-like membrane integral protein_*Zea mays*	1.29	5.159	158/1e-37	DR831450
MM20	Histone H2A_*Zea mays*	-2.4	5.521	74.7/8e-13	DR831451
MT146	Peroxisomal membrane protein_*Oryza sativa*	1.223	2.015	225/6e-58	DR831452
MT42	Ribosomal protein L2_*Eucalyptus globules*	-1.173	4.019	56/4e-05	DR831517
MM4	Inorganic phosphate transporter_*Agaricus bisporus*	-9.573	N/A	52/5e-04	DR831453
MM22	Beta tubulin_*Zea mays*	-2.84	N/A	52/7e-04	DR831454
	Defense-related				
MT4	Beta glucosidase_*Oryza sativa*	3.899	-22.1	150/2e-35	DR831570
MM2	Sulfur-rich/thionin-like protein_*Triticum aestivum*	13.251	-5.692	79.7/2e-14	DR831455
MT20	Glucan endo-1,3-beta-glucanase_*Zea mays*	2.218	-4.35	52/5e-06	DR831456
MT31	S-like RNase_*Oryza sativa*	1.801	-3.74	38.1/0.083	DR831457
MM37	Cysteine proteinase inhibitor_*Sorghum bicolor*	3.324	-2.823	93.6/2e-18	DR831459
MM76	Cysteine proteinase_*Zea mays*	2.652	-2.539	99.6/4e-18	DR831458
MT44	Polyphenol oxidase_*Triticum aestivum*	3.573	-2.228	199/4e-50	DR831460
MT177	Wilms' tumor-related protein QM_*Oryza sativa*	1.342	-2.006	120/1e-26	DR831461
MM103	Legumain-like protease_*Zea mays*	2.105	-1.945	223/4e-57	DR831462
MM79	Endo-1,4-beta glucanase Cel1_*Hordeum vulgare*	2.621	-1.829	224/2e-57	DR831463
MM78	Wound inductive gene_*Oryza sativa*	2.621	-1.763	120/2e-26	DR831464
MM86	Multiple stress responsive zinc-finger protein_*Oryza sativa*	2.428	-1.659	169/3e-41	DR831465
MM95	Oxysterol-binding protein_*Arabidopsis thaliana*	2.135	-1.647	271/4e-72	DR831466
MM71	Cytochrome P450-like protein_*Sorghum bicolor*	2.757	-1.268	365/6e-100	DR831467
MM31	Cytochrome P450 monooxygenase_ *Zea mays*	-2.253	1.803	117/1e-25	DR831468
MM73	Xa1-like protein_*Sorghum bicolor*	2.39	1.866	211/8e-54	DR831470
MT162	OTU-like cystein domain containing protein_*Oryza sativa*	-1.066	1.867	69.7/2e-11	DR831471
MT35	Cytochrome P450 monooxygenase_*Oryza sativa*	1.008	3.655	213/1e-54	DR831469
	Development				
MM65	24kDa seed maturation protein_*Oryza sativa*	3.18	-1.955	210/1e-53	DR831472
MT121	Auxin induced protein_*Saccharum*-hybrid cultivar	1.8	2.5	84.3/1e-15	DR831473
MT103	GA-induced cysteine-rich protein_*Petunia x hybrida*	-1.882	2.856	67.8/9e-11	DR831474
MT88	GH1 protein or auxin regulated protein_*Oryza sativa*	1.471	2.924	52.0/6e-04	DR831475
	Oxidative burst/stress				
MM13	Peroxidase_*Zea mays*	9.474	-11.464	244/1e-63	DR831476
MM46	Catalase_*Oryza sativa*	-2.84	-8.427	124/8e-28	DR831477
MM51	Glutathione S-transferase_*Ixodes ricinus*	3.541	-3.242	52/7e-04	DR831478
MT178	Quinone oxidoreductase_*Oryza sativa*	2.242	-2.117	334/1e-90	DR831479
MT90	Catalase isozyme3_*Zea mays*	3.605	-3.017	119/3e-26	DR831480
	Photosynthesis related				
MM23	NADP-specific isocitrate dehydrogenase_*Oryza sativa*	3.315	-3.053	233/2e-60	DR831481
MT38	Citrate synthase, glyoxisomal precursor_*Oryza sativa*	1.429	-2.87	223/2e-57	DR831482
MM60	Enolase_*Zea mays*	3.114	-2.285	187/7e-47	DR831483
MM89	Chloroplast thylakoidal processing peptidase_*Oryza sativa*	2.387	-1.486	92.8/3e-18	DR831484
MM96	RuBisco subunit binding protein beta subunit_*Zea mays*	2.231	1.005	173/1e-42	DR831485
MM56	Lipoic acid synthase_*Arabidopsis thaliana*	4	1.105	74.3/1e-12	DR831486
MM33	Type II chlorophyll a/b binding protein_*Sorghum bicolor*	-1.808	1.347	129/2e-27	DR831487
MM97	Sedoheptulose-1,7-bisphosphatase precursor_*Oryza sativa*	2.199	1.56	212/3e-54	DR831488
MM5	Cytochrome b6/f complex subunit5_*Oryza sativa*	-3.006	1.97	44.7/8e-04	DR831489
MT176	Mannose 6-phosphate reductase_*Oryza sativa*	1.339	1.992	389/6e-107	DR831490
MT152	Photosystem1 reaction center subunit2_*Oryza sativa*	-1.069	2.027	249/4e-65	DR831491
MT155	Plastid ribosomal protein L19 precursor_*Oryza sativa*	1.764	2.034	216/5e-55	DR831492

Table 2-1 *Continued*

MT151	Photosystem I chain D precursor_*Hordeum vulgare*	1.257	2.123	97.1/2e-19	DR831493
MT125	Ribosomal protein chloroplast-like_*Oryza sativa*	1.416	2.334	114/2e-24	DR831494
MT79	Photosystem2 10k protein_*Oryza sativa*	-1.08	2.558	131/1e-27	DR831568
MT23	Ferredoxin_*Zea mays*	-1.866	3.145	160/3e-38	DR831495
MM11	Chlorophyll a/b binding protein precursor_*Oryza sativa*	-2.382	3.154	224/2e-57	DR831496
MT68	29kDa ribonucleoprotein A chloroplast precursor_*Oryza sativa*	1.293	3.771	242/6e-63	DR831569
MT54	SecA-type chloroplast protein transport factor_*Oryza sativa*	1.275	3.97	164/1e-39	DR831427
MT28	Harpin induced protein_*Oryza sativa*	1.131	3.896	240/3e-62	DR831515
	Signal transduction				
MT18	Ras-GTPase activating protein binding protein2_*Oryza sativa*	11.959	-14.113	99.4/3e-20	DR831498
MT5	CCR4-NOT transcription complex subunit7_*Oryza sativa*	3.254	-7.989	43.1/0.003	DR831499
MM24	Gamma2 subunit of voltage gated Ca2+ channel_*Mus musculus*	5.657	-3.458	199/2e-50	DR831417
MM100	ADP-ribosylation factor_*Oryza sativa*	2.155	-3.053	270/3e-71	DR831500
MM19	WD domain, G-beta repeat containing protein_*Oryza sativa*	-2.172	-2.803	41/0.007	DR831423
MM41	Phospholipase_*Oryza sativa*	4.26	-2.481	252/3e-66	DR831424
MT43	Aci-reductone dioxygenase-like protein_*Oryza sativa*	1.853	-2.42	166/2e-40	DR831425
MM62	Stearoyl-acyl-carrier protein desaturase_*Oryza sativa*	2.834	-1.9	97.8/9e-20	DR831426
MM77	Steroid membrane binding protein_*Oryza sativa*	2.743	-1.472	170/1e-41	DR831428
MM107	ADP-ribosylation factor1-like_*Arabidopsis thaliana*	2.029	-1.389	198/3e-50	DR831501
MM81	Glycine-rich RNA-binding protein_*Sorghum bicolor*	2.579	-1.26	184/4e-44	DR831502
MM85	Methionine adenosyltransferase_*Hordeum vulgare*	2.444	1.302	125/6e-28	DR831503
MM82	Omega-3 fatty acid desaturase_*Zea mays*	2.585	1.348	493/e-138	DR831504
MT171	Phosphatidic acid phosphatase beta-like_*Oryza sativa*	1.066	1.794	146/4e-34	DR831505
MT166	Phosphoinositide kinase_*Oryza sativa*	1.029	1.83	310/2e-83	DR831506
MT153	GTP-binding protein typA_*Oryza sativa*	N/A	1.979	213/5e-54	DR831507
MM83	Wheat adenosylhomocysteinase-like protein_*Oryza sativa*	2.518	2.003	125/3e-28	DR831508
MT159	ARF GTPase-activating domain containing protein_*Oryza sativa*	N/A	2.007	190/2e-47	DR831509
MT143	GTP-binding protein RIC2_*Oryza sativa*	1.157	2.218	308/5e-83	DR831510
MT123	Ankyrin like protein_*Oryza sativa*	1.058	2.874	304/2e-81	DR831511
MT59	Acid cluster protein 33_*Oryza sativa*	N/A	3.202	191/6e-48	DR831512
MT65	Inorganic pyrophosphatase_*Oryza sativa*	1.207	3.461	179/6e-44	DR831513
MT63	GDSL-motif lipase/hydrolase-like protein_*Oryza sativa*	1.016	3.523	41.6/0.008	DR831514
MT37	Acyl-CoA binding protein_*Oryza sativa*	-1.241	3.939	154/2e-36	DR831516
MT26	Gamma-2 subunit of voltage-gated Ca2+ channel_*Mus musculus*	-1.493	4.147	77.4/1e-13	DR831518
MT13	Phytosulfokine receptor precursor_*Oryza sativa*	-1.607	4.807	152/4e-36	DR831519
	Unknown function				
MM7	OSJNBb0022F23.4_*Oryza sativa*	8.363	-6.945	83.2/2e-15	DR831520
MT19	Unknown_*Glycine max*	1.247	-6.238	140/3e-32	DR831521
MT21	Hypothetical protein_*Candida albicans*	2.071	-4.847	49/8e-05	DR831522
MT7	No similarity found	N/A	-4.731		DR831523
MT8	OSJNBa0016O02.6_*Oryza sativa*	N/A	-4.469	119/9e-26	DR831524
MM35	No similarity found	4.823	-1.937		DR831525
MM16	OSJNBb0014D23.16_*Oryza sativa*	5.926	-1.706	103/5e-21	DR831526
MM14	No similarity found	4.095	-1.617		DR831527
MM44	No similarity found	3.585	-1.555		DR831528
MM27	No similarity found	4.368	-1.432		DR831529
MM109	At3g26710_*Arabidopsis thaliana*	1.858	1.293	116/2e-25	DR831530
MM18	No similarity found	-3.326	1.302		DR831531
MM68	Hypothetical protein_*Sorghum bicolor*	2.892	1.715	61.2/9e-09	DR831532
MT168	No similarity found	-1.611	1.829		DR831533
MM17	No similarity found	4.955	1.852		DR831534
MT156	Unnamed protein product_*Triticum aestivum*	1.358	1.861	290/2e-77	DR831535

Table 2-1 *Continued*

MT161	Ab2-057_*Rattus norvegicus*	1.123	1.865	113/2e-24	DR831536
MT160	No similarity found	-1.134	1.888		DR831537
MT164	No similarity found	-1.14	1.913		DR831538
MT154	No similarity found	1.007	1.925		DR831539
MM99	OSJNBa0093F16.13_*Oryza sativa*	2.263	1.932	148/4e-35	DR831540
MT139	OSJNBa0017P10.11_*Oryza sativa*	1.184	1.935	97.8/9e-20	DR831541
MT149	No similarity found	1.155	2.079		DR831542
MT104	No similarity found	1.07	2.081		DR831543
MT144	No similarity found	-1.487	2.176		DR831544
MT141	Expressed protein_*Oryza sativa*	-1.364	2.216	44.7/8e-04	DR831545
MT113	No similarity found	1.358	2.305		DR831546
MT92	Unnamed protein product_*Hordeum vulgare*	1.297	2.415	189/3e-47	DR831547
MT97	No similarity found	N/A	2.486		DR831548
MT93	Unknown protein_*Oryza sativa*	-1.273	2.5	73/3e-12	DR831549
MT131	No similarity found	-1.005	2.512		DR831550
MT127	No similarity found	-1.001	2.565		DR831551
MT106	Unknown protein_*Oryza sativa*	1.194	2.726	57/2e-07	DR831552
MT105	OSJNBb0006N15.13_*Oryza sativa*	-1.285	2.834	69.3/3e-11	DR831553
MM87	Unknown protein_*Oryza sativa*	2.412	2.904	62/6e-09	DR831554
MT85	Unknown_*Saccharomyces cerevisiae*	1.014	3.145	76.6/2e-13	DR831555
MT77	Unknown protein_*Oryza sativa*	2.349	3.317	126/2e-28	DR831557
MT75	OSJNBa0081L15.5_*Oryza sativa*	-1.178	3.504	42.4/0.004	DR831556
MT72	No similarity found	1.12	4.211		DR831558
MT24	No similarity found	-1.186	4.243		DR831559
MT16	Unknown protein_*Oryza sativa*	1.845	4.608	90.9/2e-17	DR831560
MT14	No similarity found	1.259	4.611		DR831561
MT12	OSJNBa0033G05.15 *Oryza sativa*	1.264	5.053	147/2e-34	DR831562
MT173	Unnamed protein product_*Kluyveromyces lactis*	-1.266	5.367	163/2e-39	DR831563
MT3	Unknown protein_*Oryza sativa*	1.199	7.15	103/5e-21	DR831564
MT175	No similarity found	1.024	2.823		DR831567

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CHAPTER III

CHARACTERIZATION AND EXPRESSION ANALYSIS OF TWO cDNAs ENCODING DEFENSE-RELATED PROTEINS AGAINST GREENBUG FEEDING IN SORGHUM

ABSTRACT

Using suppression subtractive hybridization (SSH) and subsequent microarray analysis, expression profiles of sorghum genes responsive to greenbug phloem-feeding were previously obtained and identified. Among the profiles, two cDNAs designated MM73 and MM95 were identified to encode Xa1 and oxysterol binding protein (OSBP), respectively. Further characterization of MM73 and MM95, and the expression analyses of *Xa1* and *OSBP* genes in sorghum were performed in this study. Based on nucleotide sequences of both cDNAs, amino acid sequences were deduced and analyzed. Multiple sequence alignments of deduced amino acid sequences of MM73 (125 residues) and MM95 (142 residues) with other homologous proteins showed high similarity in amino acid sequences to Xa1 from sorghum (83%) and OSBP from Arabidopsis (84%), respectively. The expression

patterns of *Xa1* and *OSBP* genes in sorghum were analyzed using northern blot analysis. In response to three different treatments such as greenbug infestation, methyl jasmonate (MeJA) treatment, and mechanical wounding, *Xa1* and *OSBP* genes showed differential expressions exclusively bygreenbug infestation and mechanical wounding in a highly similar regulation pattern. However, MeJA treatment showed no effects on the regulation of either gene, resulting in the same levels of the expression of both genes to those showed in untreated controls. This indicates that the regulation of both genes is independent of the octadecanoid pathway involved in jasmonic acid (JA) synthesis, which is known to control diverse defense responses against insect feeding in plants.

INTRODUCTION

The greenbug (*Schizaphis graminum* Rondani) is the most serious aphid pest on important crops in the Great Plains of North America (Weng et al., 2005) and is considered a key insect pest of sorghum (*Sorghum bicolor* L.) (Tuinstra et al., 2001). Due to the continuous appearance of new greenbug biotypes, it has been difficult to control greenbug damage. Therefore ceaseless efforts in developing greenbug-resistant sorghum hybrids are being made (Porter et al., 1997). Further progress in

isolation and characterization of novel defense genes against greenbug feeding will undoubtedly potentiate a development of stable and strong greenbug-resistant sorghum cultivars by introducing the defense genes directly into high-performance cultivars using molecular gene transfer techniques.

Xa1 protein is a cytoplasmic-receptor like protein, comprised of nucleotide binding sites (NBS) with a new type of leucine-rich repeats (LRR). Xa1 is known to play a key role in defense responses against bacterial blight disease in rice, which is caused by *Xanthomonas oryzae* pv. *Oryzae* (*Xoo*) (Yoshimura et al., 1998). More than 20 genes resistant to *Xoo* were identified in rice. Unlike the *Xa21* gene encoding a LRR receptor kinase-like protein, which belongs to a different class of resistance (*R*) genes against *Xoo* in rice, the *Xa1* gene encodes a protein containing NBS-LRR motifs without a kinase domain (Iyer and McCouch, 2004). A majority of *R* genes discovered so far contain C-terminal LRRs and NBS domains. LRR and NBS are presumably involved in protein-protein interactions and signal transduction, respectively. The LRR-NBS type *R* gene family is ubiquitous in plants, plausibly suggesting that these genes are used as recognition factors against products of avirulence (*Avr*) genes from pathogens (Harris et al., 2003). The *Xa1* gene was verified to be located in chromosome 4 in rice, and sequence analyses of rice chromosome 4 revealed that a cluster of *Xa1* genes, comprising six members, is

located in the chromosome (Feng et al., 2002). Application of the chemical probenazole which is known to elicit systemic acquired resistance (SAR), and is used for prevention of rice blast disease induces expression of the *RPR1* gene containing NBS-LRR motifs. The *RPR1* gene shares a major structural similarity with the *Xa1* gene and both genes are classified into the same *R* gene class. In addition, both genes are induced by inoculation with rice blast fungus (Sakamoto et al., 1999). Based on this result, Xa1 may thus be involved in induction of SAR in plants. The rice *Pib* gene is known to be one of the *R* genes against rice blast disease, caused by the infection of fungus *Magnaporthe grisea*. The *Pib* gene consists of NBS-LRR motifs similar to structures of other *R* genes, including the *Xa1* (Wang et al., 1999).

Oxysterols, a group of 27-carbon oxygenated derivatives of cholesterol, play an important role in regulation of the expression of specific genes by serving as ligands, which bind to the receptors on nuclear membranes (Edward and Ericsson, 1999). A potato gene encoding an oxysterol binding protein (*StOBP1*) was quickly up-regulated by the infection of fungus *Phytophthora infestant*. The *StOBP1* was induced by oligogalacturonides generated by pectinase attacks on plant cell walls, and was revealed to be elicited by an independent pathway from other resistance (*R*) gene-mediated defense events (Avrova et al., 2004). Cytochrome P450 steroid hydroxylase (CYP90) plays an important role in the biosynthesis of brassinosteroid and in defense

against diverse stresses, including insect feeding, in plants. CYP90 was verified to interact with oxysterol binding protein (OSBP) to control activities of membrane-bound steroid regulatory machineries (Salchert et al., 1998). It is believed that OSBP suppresses sterol biosynthesis through interactions with oxysterols, and also plays a crucial role in controlling Golgi function through regulation of the adenine diphosphate-ribosylation factor (ARF) cycle (Li et al., 2002). OSBP contains a ligand binding (LB) domain, which interacts with oxysterols. OSBP perceives conformation of ligands, and delivers signals downstream (Lehto and Olkkonen, 2003). OSBP also contains a pleckstrin homology (PH) domain in the amino-terminal region interacting with phosphatidylinositol lipids, and thereby moves to Golgi membranes where the phosphatidylinositol lipids are abundant (Beh et al., 2001).

From our previous study (Park et al., 2005), a total of 157 different genes verified to be differentially regulated in response to greenbug feeding were profiled through suppression subtractive hybridization (SSH) and subsequent microarray analyses. In this study, we isolated and characterized two cDNAs encoding sorghum Xa1 and OSBP, which are involved in defense responses against greenbug phloem-feeding. Based on the deduced amino acid sequences of MM73 and MM95, multiple sequence alignments with several homologous proteins from other species were performed. Sequence analyses were also performed using on-line programs to

determine the structures of two cDNAs and their putative encoded proteins. In addition, the expression patterns of *Xa1* and *OSBP* genes in sorghum in response to three different treatments such as greenbug infestation, methyl jasmonate (MeJA) treatment, and mechanical wounding were investigated to understand regulatory mechanisms of both genes using northern-blot analysis.

MATERIALS AND METHODS

Plant material and greenbug growth conditions

Sorghum (*Sorghum bicolor* L.) seeds from M627, a highly greenbug resistant line, (http://www.dowagro.com/webapps/Include/GetDoc.aspx?ObjectId=&filepath=mycogen/pdfs/noreg/010-10899.pdf) were planted in Absorb-N-Dry soil (Bacones Mineral Corp., Flaonia, TX) in plastic pots (6 inch diameter and 5.5 inch depth). The seeds were provided by Mycogen Seeds (Indianapolis, IN). Seedlings were grown in a greenhouse for 10 days at 30℃ and 60% relative humidity in a 14 h-light/10 h-dark photoperiod. After seeds were germinated, a transparent plastic cage with three air vents was put over each pot to protect seedlings from unwanted herbivory. Biotype I greenbugs were reared on barley seedlings in a growth chamber for 10 days at 30℃

and 60% relative humidity in a 14 h-light/10 h-dark photoperiod. Biotype I is reported to be currently the most widespread greenbug biotype in U.S. (Tuinstra et al., 2001).

Plant treatments

For all treatments, 10-day-old sorghum seedlings were used and untreated control sorghum seedlings (C) were collected at the same time with other treated plant samples. Greenbugs were transferred to seedlings using a paint brush. Approximately 30 greenbugs were confined on each seedling. Greenbugs were removed from seedlings after 6, 12, 24, 48, and 72 h time points following greenbug infestation. Collected seedlings were frozen immediately in liquid nitrogen, and stored at -80℃ prior to use. The wounding treatment was accomplished by scratching the surfaces of leaves and stems (more than 50% of seedling surfaces) using a sterilized file. The MeJA treatment was conducted as follows. A MeJA solution (Aldrich, Milwaukee, WI) was added to distilled water to a final concentration of 200 μM, and the diluted solution was sprayed sufficiently until seedlings were drenched using a spray bottle. The MeJA treatment was performed in an isolated area of the greenhouse to prevent unwanted spread of MeJA volatiles. After wounding and MeJA treatment, seedlings were collected at the same time points (6, 12, 24, 48, and 72 h) as seedlings treated

with greenbugs were collected.

DNA sequencing and database search

The cDNA clones showing differential expressions against greenbugs were obtained from our previous study (Park et al., 2005) using suppression subtractive hybridization (SSH) and subsequent microarray analysis. Sequencing of the cDNA clones was performed using the BigDye™ terminator sequencing kit (Applied BioSystem, Foster City, CA) and ABI Model 3700 DNA Analyzer (Applied BioSystem). The database search was performed on the basis of cDNA sequences using BLASTX and BLASTN. BLASTN was used in case of the absence of any matched hits when performing BLASTX. All cDNA sequences were submitted to the GenBank dbEST and corresponding accession numbers were assigned.

Sequence analysis

Amino acid sequences were deduced based on the cDNA sequences and analyzed using the programs such as Translate and ProtParam provided from the ExPASy (Expert Protein Analysis System) proteomics server (http://us.expasy.org). The

deduced amino acid sequences were used to search for homologous proteins using BLASTP, and then amino acid sequences of matched hits were obtained in FASTA format and used for multiple sequence alignments using the ClustalW on-line program at http://www.ebi.ac.uk/clustalw/. Phylograms were produced using the CLC Free Workbench program version 2.01 (CLC bio, Aarhus, Denmark).

Northern-blot analysis

Total RNA was isolated from seedlings collected after the five different time points (6, 12, 24, 48, and 72 h) of each treatment applied to seedlings, respectively. Seedling tissues were ground into a fine powder in liquid nitrogen, and total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Ten micrograms of total RNA from each sample was fractionated in a 1% agarose gel containing 1.1 M formaldehyde, and then transferred to Hybond-N^+ membrane (Amersham Biosciences, Piscataway, NJ) using the alkaline solution (3 M NaCl and 0.01 N NaOH) transfer method. Probes were produced by rescuing the subtractive cDNA inserts, previously ligated to the vector pCR2.1, by PCR amplification using primers 5'-TCGAGCGGCCGCCCGGGCAGGT-3' (Nested 1, Invitrogen) and 5'-AGCGTGGTCGCGGCCGAGGT-3' (Nested 2R, Invitrogen) and

labeled with of ^{32}P-dCTP (Perkin-Elmer, Wellesley, MA) and hybridized to pre-warmed membranes soaked with 2 ml of the UltraHyb buffer (Ambion, Austin, TX) at 42℃ overnight. Membranes were washed with 2X SSC and 0.1% SDS at 65℃ for 20 min, and then with 0.1X SSC and 0.1% SDS at 60℃ for 10 min. Washed membranes were exposed to Kodak BioMax MS film (Kodak, Rochester, NY) at -80℃ overnight.

RESULTS AND DISCUSSION

Isolation and characterization of two cDNA clones encoding Xa1 and OSBP

In our previous work (Park et al., 2005), 157 different cDNAs identified to be responsive to greenbug phloem-feeding in sorghum were obtained using SSH and subsequent microarray analysis. Among the expression profiles obtained, two cDNAs designated as MM73 (377 bp) and MM95 (429 bp) were verified to encode Xa1 and OSBP, respectively, by database searches. Sequences of both cDNA clones were submitted to the GenBank dbEST (accession numbers; MM73: DR831470, MM95: DR831466). Based on the nucleotide sequences, amino aid sequences were deduced for the two cDNAs. The product of MM73 consists of 125 residues, highly enriched in leucine (12.9%), and the product of MM95 consists of 142 residues, enriched in a

neutral amino acid, valine (11.3%) (Fig. 3-1, A and B). Using the BLASTX search, MM73 was identified to encode a protein highly homologous to Xa1 from sorghum (82%), and the MM95 encoded a protein showing a high identity to OSBP from Arabidopsis (84%).

On the basis of the deduced amino acid sequences of MM73 and MM95, multiple alignments with amino acid sequences of other homologous proteins obtained from the GenBank databases were performed using ClustalW (Fig. 3-2, A and B). As shown in Table 3-1, the amino acid sequence of MM73 shared 83% identity to Xa1 from sorghum (AAO16692), 44% to Xa1 from rice (BAD29495), and 40% to NBS-LRR (NL) disease-related protein from barley (CAD45028). The amino acid sequence of MM95 showed 84%, 83%, 80%, and 80% identities with OSBPs from *Arabidopsis thaliana* A.t_OSBP2 (AAN15434), A.t_OSBP1 (CAB82983), A.t_OSBP3 (AAT14027), and potato (AAR25799), respectively. In addition, phylogenetic trees were generated based on amino acid sequences of Xa1 and OSBP from various organisms, as well as of MM73 and MM95, to assess evolutionary proximities among the homologous proteins using the neighbor joining method CLC Free Workbench program, respectively (Fig. 3-3). The MM73 showed a close proximity to Xa1 from sorghum, but was distantly related to Xa1s of rice and barley in terms of evolutionary changes introduced in amino acid sequences. The close

relation of MM95 to OSBPs from Arabidopsis and potato was reflected by high identity in amino acid sequences in contrast to a more distant relation to OSBPs from amphibian and canine.

Expression patterns of *Xa1* and *OSBP* genes in sorghum plants

To investigate the regulation patterns of *Xa1* and *OSBP* genes in sorghum, northern blot analyses were performed using total RNA isolated from sorghum seedlings subject to three different treatments such as greenbug infestation, MeJA treatment, and mechanical wounding. It is known that plants utilize several universal molecular regulators, including salicylic acid (SA), jasmonic acid (JA), abscisic acid (ABA), and ethylene (ET) to control the expression of genes involved in defense responses against diverse stresses by crosstalk among the molecular regulators, antagonistically or synergistically (Doarse et al., 1995; Reymond and Farmer, 1998). Insect feeding on plants resulted in marked changes in gene expression, which are believed to be orchestrated by SA, JA, ET, and reactive oxygen species (ROS) (Walling, 2000; Huang, 2005). Comparison of transcriptional profiles obtained individually from Arabidopsis treated by mechanical wounding and insect feeding showed considerable differences (Reymond et al., 2000). In addition, the results showed that water stress-

induced genes were more highly induced by mechanical damage than by insect feeding, indicating the existence of specific insect-feeding machineries able to avoid activation of plantdefense responses.

To understand regulation mechanisms of gene expression in response to greenbug phloem-feeding in sorghum, the expression patterns of *Xa1* and *OSBP* genes in response to three different treatments were analyzed and compared using northern-blot analyses. To determine effects of the well-known plant molecular regulator jasmonic acid (JA), sorghum seedlings were subject to the MeJA treatment in addition to the introduction of greenbug infestation and mechanical wounding. Then, the expression patterns of *Xa1* and *OSBP* genes in response to these three different treatments and untreated controls were investigated at five different time points (6, 12, 24, 48, and 72 h). The results showed that both *Xa1* and *OSBP* genes were constitutively expressed in the sorghum line M627 used in this study. The *R* genes are usually expressed constitutively in untreated plants. The *Ha-NTIR11g* gene encoding the R protein comprised of coiled-coil domain (CC)-NBS-LRR motifs was verified to be constitutively expressed in sunflower, and other *R* genes, including the *RPM1*, were also found to be constitutively expressed in Arabidopsis (Grant et al., 1995; Radwan et al., 2005). In a few cases, the expression of some *R* genes including the *Xa1*, are differentially regulated bvarious stresses (Yoshimura et al., 1998; Levy et

al., 2004). In our results, both *Xa1* and *OSBP* genes were differentially regulated exclusively by greenbug infestation and mechanical wounding, in a similar pattern. In contrast, the MeJA treatment did not affect the expression of either gene, and resulted in appearance of the same expression patterns of the genes to those shown by the controls (Fig. 3-4).

After 6 h of each treatment, expression patterns of both genes responding to all treatments (including the untreated control) were nearly identical to each other, but the expressions of both genes were suppressed exclusively by greenbug infestation and mechanical wounding at 12 h. This suppression was reversed to induction at 24 h, but the levels of expression of both genes were still a little weaker in greenbug infested and mechanically wounded seedlings, as compared to the expression levels in the control and the MeJA treated seedlings. However, this suppression by greenbug infestation and wounding suddenly reverted to intense induction at 48 h and maintained a high level of induction at 72 h. In contrast, the expressions of both genes in the untreated control and MeJA treated seedlings were abruptly suppressed at 48 h in contrast to the high induction by greenbug infestation and mechanical wounding. This suppression converted to induction at 72 h, but the expression levels of both genes in the control and MeJA treated were still a little lower than those in the greenbug infested and mechanically wounded. It is plausible that this suppression in

the control is attributed to developmental regulation of the genes in sorghum. Our previous findings showed that the expression patterns of genes encoding caffeic acid *O*-methyltransferase (*COMT*), proline-rich protein (*PRP*), and glycosyltransferase (*GT*) were strikingly similar to those of *Xa1* and *OSBP* (manuscript in preparation). The soybean *PRP* genes were corroborated to be differentially regulated by development and organ specificity (Hong et al., 1989). The *COMT* gene and the gene encoding caffeoyl CoA 3-*O*-methyltransferase (*CCOMT*) were verified to be developmentally regulated in alfalfa (Inoue et al., 1998). The expression of both *COMT* and *CCOMT* genes showed a nearly identical expression pattern in stems and roots of alfalfa. Differential expression of the *PsUGT1* gene -encoding UDP-glucuronosyltransferase by development was demonstrated in pea (Woo et al., 1999). Inhibition of the expression of *PsUGT1* gene by the inducible expression of *PsUGT1* antisense mRNA resulted in mortality in both pea and alfalfa due to the complete prevention of root development. It seems that the expression of these genes, including the *Xa1* and *OSBP*, were controlled by common regulators in sorghum.

From the results of expression analyses, the expressions of *Xa1* and *OSBP* genes were verified to be independent of JA, which is a well-known molecular regulator involved in defense responses against various stresses, including insect feeding. This implies that both genes were regulated via pathways independent from

the octadecanoid pathway which is involved in JA biosynthesis. Definite causes of differential expression of these genes by greenbug feeding, and defensive contributions of Xa1 and OSBP against greenbugs have remained elusive. However, it was confirmed here that the expressions of both genes were regulated in a similar pattern by greenbug infestation and mechanical wounding. Therefore, future studies for elucidation of the regulation mechanisms of *Xa1* and *OSBP* gene will be focused on finding common *cis*- and *trans*-acting elements in the promoter regions of both genes in response to greenbug infestation and wounding. In addition, production of sorghum mutants manipulated to be inhibited the respective expressions of *Xa1* and *OSBP* genes will provide more detailed information on the functional roles of both genes in plant defense responses.

In conclusion, a collection of cDNAs identified to respond to greenbug phloem-feeding in sorghum was isolated using SSH and microarray analysis. Among these cDNAs, two cDNAs designated to MM73 and MM95 were further characterized. The deduced proteins of cDNAs designated MM73 (377 bp) and MM95 (429 bp) consist of 125 and 142 residues, respectively. MM73 and MM95 were identified to encode Xa1 and OSBP, respectively, and confirmed to show high identity in amino acid sequences with homologous proteins from other species using multiple alignments. Using northern-blot analysis, the expressions of *Xa1* and *OSBP* genes

were verified to be regulated independently from JA, which is involved in the regulation of a majority of defense genes against insect feeding in plants. In addition, the expression of both genes, *Xa1* and *OSBP*, were differentially regulated in response to greenbug infestation and mechanical wounding, in a highly similar pattern. In contrast, the MeJA treatment showed no effects on regulation of the expression of both genes.

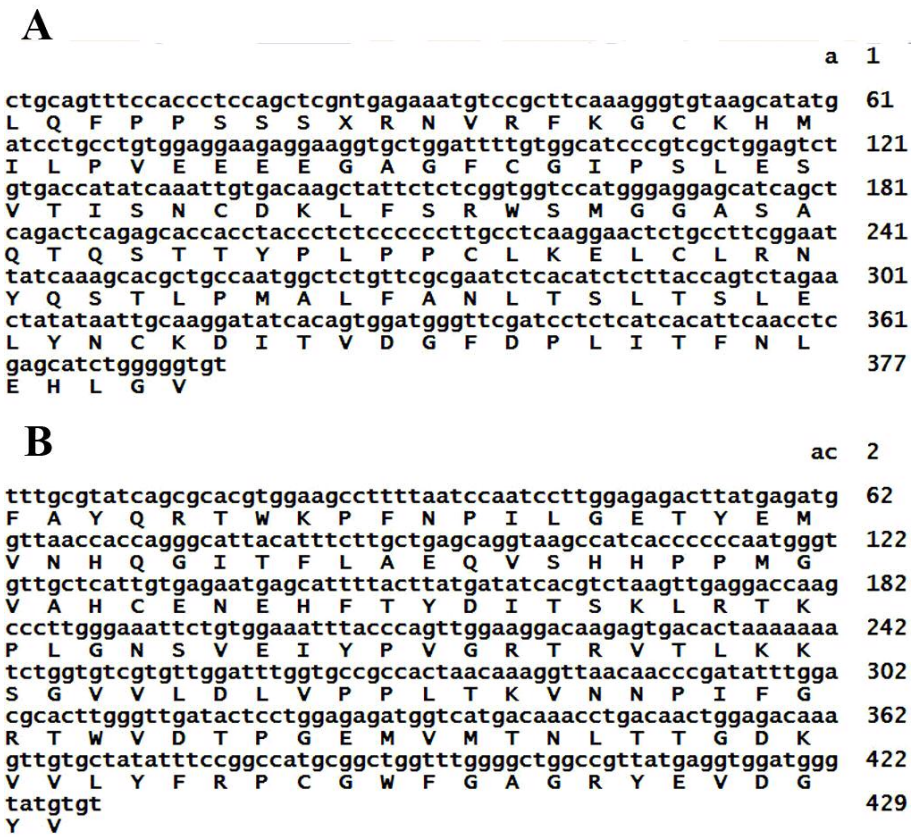

Fig. 3-1 Nucleotide and deduced amino acid sequences of MM73 (A) and MM95 (B). The deduced amino acid sequences are shown below the first nucleotide of each corresponding codon.

A

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MM73          ---------------------------LQFPPSSSXRNVRFKGCKHMILPVEEEE-GAGF 32
Sorghum       SNLFKCFPALSRLDVSTSSDEDHEEVVLQFPPSSSLRNVCFNRCKNMILPVEAEE-GAGF 1021
Rice          TKLLNRFPALTEFHLIFSSFEVGEEAVLQLPSSNLLSYVRIWCCKNLVLPVAD---GGGL 1135
Barley        SKVLNCFPAVSVLEIVGEGNHDYEKYVMQLPSSSSLQKLSFRGCNGLVLVPAEKENGGGI 1137
                                      :*:*.*.    : :  *: ::*       *.*:

MM73          C-GIPSLESVTISNCDKLFSRWSMG--GASAQTQSTTYPLPPCLKELCLRNYQSTLPMAL 89
Sorghum       R-ALPSLESVTVINCDKLFSRWSMG--GAAAQTQSTIYPLPPCLKELCLCYQQSTLPMAL 1078
Rice          H-DLSSLQEVEIRGCGKMFDRCS-----MVEAGARSNKFFPASLRELNISDELSIQSMAL 1189
Barley        EEDNSLLQSLTISRCGKLLCLWPSMGMGMGESAETIIYPFPASLRKLYVQEETSIKSMAV 1197
                  . *:.: :  *.*::   .                :*..*::* :    *  .**:

MM73          FANLTSLTSLELYNCKDITVDGFDPLITFNLEHLGV------------------------ 125
Sorghum       FANLTSLTNLELYNCKDITVDGIDPHITFNLEQLEVYNWRDGEAEPYSVAADLLAAVART 1138
Rice          LTNLTSLTHLTLINCDNLTVHGFDPLITCSLKELVVYKKADDEIHLYSLADDLFLEVATR 1249
Barley        LSNLTSLTSLSLSYCRNLTVDGFNPLMAVNLIQLHVQCG-------KTLAADLLSQVASH 1250
              ::****** * *  * ::**.*::* :: .* .* *
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B

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MM95          -----------------FAYQRTWKPFNPILGETYEMVNHQGITFLAEQVSHHPPMGVAH 43
A.t_OSBP1     DPYMRMVYASSWAISVYYAYQRTWKPFNPILGETYEMTNHNGINFIAEQVCHHPPMSAGH 155
A.t_OSBP2     DPYLRLVYASSWAISVYYAFQRTWKPFNPILGETYEMVNHGGISFISEQVSHHPPMSAGH 142
A.t_OSBP3     DPYLRMVYASSWAISVYYAFQRTWKPFNPILGETYEMANYNGVNFISEQVSHHPPMSAGH 154
Potato        DPHMRLVYAATWFISLYHALQRTWKPFNPILGETYEFVNHAGITFIAEQVCHHPPIGAAH 180
                        .* ****************:.*: *:.*::***.****:...*

MM95          CENEHFTYDITSKLRTKPLGNSVEIYPVGRTRVTLKKSGVVLDLVPPLTKVNNPIFGRTW 103
A.t_OSBP1     AENEHFAYDCTSKLKTKFLGNSIDVYPVGRTRVTLKRDGVVLDLVPPLTKVHNLIFGRTW 215
A.t_OSBP2     AENEHFIYDITSKLKTKLLGNSVDVYPVGRTRVTLKKDGVVLDLVPPLTKIHNLIFGRTW 202
A.t_OSBP3     AENEHFTYDCTSKLKTKFLGNSIDVYPVGRTRVTLKRDGVVLDLVPPLTKVHNLIFGRTW 214
Potato        AENEHFKYDITSKVKSKFLGNSVEVYPLGRSRLTLKKSGVVLDLVPPPTKVHNLIFGRTW 240
              .***** ** ***:::* ****:::**:**:*:***:.********* **::* ******

MM95          VDTPGEMVMTNLTTGDKVVLYFRPCGWFGAGRYEVDGYV---------------------- 142
A.t_OSBP1     VDSPGEMVMTNLTTGDKVVLYFQPCGWFGSGRYEVDGYVYNSAEEPKMLMTGKWNESLSY 275
A.t_OSBP2     VDSPGEMVMTNLTTGDKVVLYFQPCGWFGSGRYEVDGYVYSAAEEPKIMMTGKWNEKMSY 262
A.t_OSBP3     VDSPGEMIMTNQTTGDKVVLYFQPCGWFGSGRYEVDGYVYNASEEPKILMTGKWNESMSY 274
Potato        IDSPGEMILTNLTTGDKVLLYFQPCGWFGAGRYEVDGYVYNSEEEPKILMTGKWNESISY 300
              :*:****::** ******:***:******:**********
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Fig. 3-2 Alignment of amino acid sequences of MM73 (A) and MM95 (B) with Xa1 and OSBP proteins from diverse species, respectively. Residues identical in all proteins compared are marked by asterisks, and residues showing similarity are denoted by periods and colons. The GenBank accession numbers assigned to the sequences analyzed are as follows: sorghum (AAO16692), rice (BAD29495), barley (CAD45028), A.t_OSBP1 (CAB82983), A.t_OSBP2 (AAN15434), A.t_OSBP3 (AAF14027), and potato (AAR25799). The amino acid sequences of MM73 and MM95 used in the alignments were deduced from nucleotide sequences.

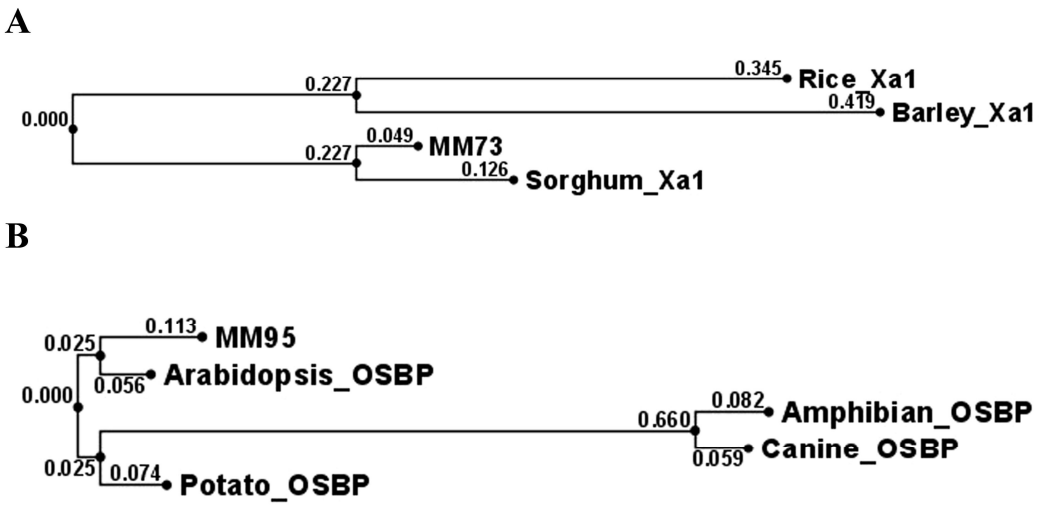

Fig. 3-3 Phylogenetic trees deduced from amino acid sequences of Xa1 (A) and OSBP (B) from diverse species produced using the neighbor joining method ClustalW (version 1.82). The branch lengths in phylograms are proportional to the amount of inferred evolutionary change. Branch length values are shown. The accession numbers of the sequences used in the phylograms were: sorghum (AAO16692), rice (BAD29495), barley (CAD45028), Arabidopsis (AAN15434), potato (AAR25799), amphibian (NP_991401), and canine (XP_537881). The amino acid sequences of MM73 and MM95 used in phylogenetic analyses were deduced from nucleotide sequences.

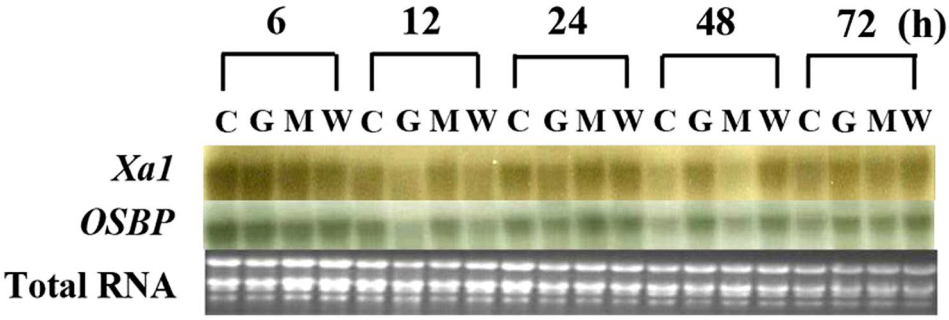

Fig. 3-4 Expression analysis of *Xa1* and *OSBP* genes. Total RNA was isolated from sorghum M627 seedlings subject to three different treatments (G, greenbug infestation; M, MeJA treatment; W, mechanical wounding) at five different time points (6, 12, 24, 48, and 72 h). Untreated controls (C) collected at the same time points were also compared. Equal amounts of RNA loading was evidenced by intensity of total RNA bands. *Xa1*, Xa1-encoding gene; *OSBP*, oxysterol binding protein-encoding gene.

Table 3-1 Sequence identities in deduced amino acid sequences of MM73 and MM95 with homologous proteins. [a]cDNAs identified to encode Xa1 and OSBP. [b]NL indicates NBS-LRR disease-related protein. A.t indicates *Arabidopsis thaliana*. [c]Homology for each pair of sequences was shown as a percent score.

SeqA Name[a]	Length (aa)	SeqB Name[b]	Length (aa)	Score[c] (%)
MM73	125	Sorghum_Xa1	1284	83
MM73	125	Rice_Xa1	1394	44
MM73	125	Barley_NL	1366	40
MM95	142	A.t_OSBP1	404	83
MM95	142	A.t_OSBP2	392	84
MM95	142	A.t_OSBP3	404	80
MM95	142	Potato_OSBP	459	80

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VITA

SUNG-JIN PARK

Candidate for the Degree of

Doctor of Phliosophy

Thesis: IDENTIFICATION OF EXPRESSION PROFILES OF SORGHUM GENES IN RESPONSE TO GREENBUG PHLOEM-FEEDING & FURTHER CHARACTERIZATION OF THE TWO SORGHUM DEFENSE-RELATED GENES, *Xa1* AND *OXYSTEROL BINDING PROTEIN*

Major Field: Plant Science

Biography:

Education: Received Bachelor of Arts in Crop Science from Korea University, Seoul, Korea in August 1997; received Master of Science in Molecular Biology from Korea University, Seoul, Korea in August 1999. Complete the Requirements for the Doctor of Philosophy degree at Oklahoma State University in December, 2005.

Experience: Full Scholarship, Korea University, Seoul, Korea, 1997-1999; Aventis Pharma Co., LTD, Seoul, Korea, 2001-2002; Graduate Research Assistant, Oklahoma State University, Stillwater, OK, U.S.A, 2002-2005.

Name: Sung-Jin Park Date of Degree: December, 2005

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: IDENTIFICATION OF EXPRESSION PROFILES OF SORGHUM GENES IN RESPONSE TO GREENBUG PHLOEM-FEEDING & CHARACTERIZATION OF THE TWO SORGHUM DEFENSE-RELATED GENES, *Xa1* AND *OXYSTEROL BINDING PROTEIN* GENES

Pages in Study: 96 Candidate for the Doctor of Philosophy

Scope and Method of Study: The greenbug phloem-feeding elicits unique interactions with plants. Using suppression subtractive hybridization (SSH) and subsequent microarray analyses, expression profiling of sorghum genes in response to greenbug feeding was performed to understand a regulation mechanism of molecular defense responses against greenbug phloem-feeding in sorghum. Among the expression profiles, two genes identified to encode Xa1 and Oxysterol binding protein (OSBP) were further characterized, and their regulatory mechanisms investigated by comparison of expression patterns of the two genes responsive to three different conditions such as greenbug infestation, methyl jasmonate treatment, and mechanical wounding.

Findings and Conclusions: A total of 157 genes identified to respond greenbug feeding was obtained by SSH and microarray analyses. These genes were classified into nine categories according to their metabolic functions. Several molecular regulators such as jasmonic acid, salicylic acid, abscisic acid, auxin, and gibberellic acid were involved in regulation of defense responses against greenbugs in sorghum. In our profiles, several genes which have not been reported in their roles in defense responses against greenbugs were obtained, including the genes encoding Xa1 (*Xa1*) and oxysterol binding protein (*OSBP*). Multiple aligning of deduced amino acid sequences of the two genes with other homologous proteins from other species showed high identity in amino acid sequences. Expression analyses of *Xa1* and *OSBP* genes showed that regulations of both genes were not affected by MeJA, but conducted via independent pathways. The expression patterns of both genes showed high similarity between greenbug infested and mechanically wounded sorghum seedlings.

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